DESCRIPTION

GERM-RESPONSIVE PROMOTER

TECHNICAL FIELD

The present invention relates to a promoter that is responsive to pathogen, and also to pathogen-resistant plants utilizing the promoter.

BACKGROUND ART

There lies an apparent race-specific parasitogenesis between potato Phytophthora infestants and potato plants. Such a specific relationship between a host and a pathogen is typically determined by the combination of avirulent genes contained in the pathogen and true resistant genes contained in the host. Dynamic resistance response is induced in the host when it is experiencing infection with an incompatible race. Specifically, production of reactive oxygen species, death of hypersensitive cell, production of phytoalexins (for potatoes, rishitin), expression of PR (Pathogenesis-Related) protein, formation of papilla, lignification and other resistance responses are triggered in the infected tissue, thereby arresting further development of the pathogen (See references 15, 32, 44, 45, and 47). In contrast, these resistance responses fail in the process of infection with a compatible race, allowing pathogen penetration and resulting in lethal, systemic infectious disease of potato.

One of the most important and local resistance responses among the dynamic resistance responses referred to above is thought to be accumulation of phytoalexin. Phytoalexins are small molecule compounds with microbiocidal action induced to accumulate upon pathogen infection, and has been indicated to be a crucial element in successful infection (See references 12, 13, 21, 28, and 46). Phytoalexins for potato are sesquiterpenoid compounds that are synthesized in isoprenoid metabolic system (Fig. 1).

It is known that isoprenoid synthesis in potato is rapidly converted from that for sterol/glycoalkaloid synthesis to that for sesquiterpenoid phytoalexin synthesis upon treatment with elicitor or inoculation of any incompatible race. This phenomenon is thought to be regulated by squalene synthase and sesquiterpene cyclase

that act coordinately in the rate-limiting step of isoprenoid synthesis system to diverge the pathway into sterol glycoalkaroid synthesis and isoprenoid phytoalexin synthesis, respectively (See reference 8). Sesquiterpene cyclase for potato is vetispiradiene synthase and designated as potato vetispiradiene synthase (PVS) (See Reference 53). It has been reported that the PVS activity in potato tuber is significantly increased by inoculating pathogen or treating with HWC, which is derived from potato pathogen (See Reference 54). In addition, it is known that such an elicitor treatment leads to activation of sesquiterpenoid synthesis pathway also in tobacco plants, resulting in production of capsidiol, a type of phytoalexin (See References 42 and 48). Recently, these phenomena have been elucidated at the level of gene expression. As a result of Northern analysis of RNA extract from potato tuber using cDNA of PVS and squalene synthase isolated from potato as probes, it was found that PVS mRNA was temporarily induced to accumulate in the area where compatible and incompatible races had been inoculated. On the other hand, it has been shown that accumulation of mRNA for squalene synthase induced in the area of wound is suppressed by inoculation with compatible and incompatible races (See Reference 53). However, this report is not consistent with the observation that only the inoculation with incompatible race leads to biosynthesis of phytoalexin to arrest development of pathogen (See Reference 40).

It is generally known that most of plant genes constitute multigene families, with each isogene having discrete organ specificity and metabolic action in response to stimuli. It has been reported that PVS genes in potato plants also constitute multigene families having PVS 1 through 4 members (See Reference 53). Details of their behavior in expression are yet to be known.

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DISCLOSURE OF INVENTION

Attempts have been made to enhance disease resistance of plants by utilizing their resistance response. One of such efforts seeks to use pathogen responsive promoter to trigger production of resistance inducer specifically at the time of disease infliction. In this method, an effective protection is provided by resistance inducer specifically and promptly produced in response to disease infliction.

Several pathogen responsive promoters have been discovered in the past, most of which are inducible not only on the occasion of pathogen infection but also of wound or at sites under normal course of growth. Accordingly, if such a promoter is used to introduce a transgene, wich is involved in production of resistance inducer, into a plant, the resultant modified plant will have the transgene expressed not only at

the time and location of infection, but harmfully elsewhere as well. The present invention is made, based on the above understanding, in order to provide a promoter that is responsive specifically to pathogen infection (pathogen responsive promoter) and method for producing pathogen resistant plants utilizing the same.

In view of the objectives described above, the present inventor first sought to obtain a promoter that is responsive specifically to the notorious late blight pathogen, Phytophthora infestans, a devastating disease of potato plants. Potato vetispiradiene (PVS) is a one of those genes involved in production of phytoalexin. The inventor first investigated the expression trend of each of the PVS members (PVS1 to PVS4) in leaf tissue which is a primary infection site of P. infeatans, and found that only PVS3 was significantly induced after inoculation with either compatible and incompatible race. Thus, it was demonstrated that the promoter for PVS3 is responsive also to infection with compatible race.

Genomic library of potato was then constructed to sequence PVS3. After several rounds of screening, genomic DNA sequence of PVS3 was successfully determined, and PVS3 promoter region and its function predicted, thus confirming the responsiveness to P. infeatans. In order to further explore the function of the deduced promoter region, potato transformant was constructed which had the deduced promoter region inserted upstream of GUS gene, and a panel of study conducted using the transformant. It was found that leaf resection (wound) did not evoke GUS staining, whereas inoculation with compatible race of P. infeatans did. In conclusion, the promoter region was determined to be Phytophthora-specific, i.e. to have pathogen-specific responsiveness.

As described above, the present inventor has successfully obtained a promoter that is responsive specifically to pathogen (pathogen-responsive promoter). The present promoter will allow creation of a plant that expresses a gene of interest only when infected with pathogen. Specifically, in such a transformant having the present promoter along with a transgene, the introduced promoter will induced specifically to express the transgene. If the transgene is the one that activates protective response, the resultant plant will have its protective response activated specifically in response to pathogen infection. Accordingly, the plant will be highly resistant to pathogen infection.

Gene transcription, in general, begins with binding of a protein called transactivator to a particular sequence consisting of several to ten and several bases called cis-sequence within promoter regions (Reference 66). Identification of cis-sequences is therefore a first step of elucidation of transcription mechanism. Based on such an understanding, the present inventor designed a panel of identified PVS3 promoter sequences with varied portions deleted, combined them with GUS gene to produce chimeric PVS3:GUS, and temporarily introduced them into leaf tissue to examine PVS3 promoter activity. As a result, it was successfully found a region consisting of 50 bp (SEQ ID NO: 23), and identified it as a region essential for PVS3 promoter activity. Besides, any known regulation motif was not found in the region at this time point.

Although the pathogen-responsive promoter (PVS3 promoter) which the present inventor successfully identified had been derived from potato plant, the promoter may be applicable to plants other than potatoes. First, while PVS3 is an enzyme that catalyzes synthesis of phytoalexin in potato plants, phytoalexin of eggplant, Solanaceae, is similarly terpene compound, and has a common synthesis pathway. In addition, the present gene is SIPK and WIPK dependent inducible, as will be described later in detail. It is generally known that these two enzymes are involved in protective response of a number of plants, including Solanaceae. In view of these similarities, the present promoter (PVS3 promoter) is expected to be applicable as pathogen-responsive promoter not only to Solanacease but also Brassicaceae (See Reference 57), Leguminosae (See Reference 58) and other wide variety of plants having reported relevance of SIPK and WIPK orthologs.

The present invention is based on the above study and findings, and provides:

[1] A pathogen-responsive promoter, comprising:

(a) a DNA comprising nucleotide sequence shown in SEQ ID NO:1;

(b) a DNA comprising nucleotide sequence shown in SEQ ID NO:1 with replacement, deletion, insertion or addition of one or more nucleotide(s) and functioning as pathogen-responsive promoter in plant cell; or

(c) a DNA hybridizing to DNA of (a) or (b) under a stringent condition and functioning as pathogen-responsive promoter in plant cell.

[2] A pathogen-responsive promoter, comprising:

(A) a DNA comprising nucleotide sequence shown in SEQ ID NO:2;

(B) a DNA comprising nucleotide sequence shown in SEQ ID NO:2 with replacement, deletion, insertion or addition of one or more nucleotide(s) and functioning as pathogen-responsive promoter in plant cell; or

(C) a DNA hybridizing to the DNA of (A) or (B) under a stringent condition and functioning as pathogen-responsive promoter in plant cell.

[3] A pathogen-responsive promoter, comprising:

(1) a DNA comprising a continuous portion of nucleotide sequence shown in SEQ ID NO:1, and functioning as pathogen-responsive promoter in plant cell;

(2) a DNA according to (1) with replacement, deletion, insertion or addition of one or more nucleotide(s) and functioning as pathogen-responsive promoter in plant cells; or

(3) a DNA hybridizing to the DNA of (1) or (2) under a stringent condition and functioning as pathogen-responsive promoter in plant cell.

[4] A pathogen-responsive promoter, comprising:

(i) a DNA comprising nucleotide sequence shown in SEQ ID NO:22;

(ii) a DNA comprising nucleotide sequence shown in SEQ ID NO:22 with replacement, deletion, insertion or addition of one or more nucleotide(s) and functioning as pathogen-responsive promoter in plant cell; or

(iii) a DNA hybridizing to the DNA of (i) or (ii) under a stringent condition and functioning as pathogen-responsive promoter in plant cell.

[5] A pathogen-responsive promoter functioning as pathogen-responsive promoter in plant cell and comprising:

(I) a DNA comprising nucleotide sequence shown in SEQ ID NO:23;

(ii) a DNA comprising nucleotide sequence shown in SEQ ID NO:23 with replacement, deletion, insertion or addition of one or more nucleotide(s); or

(iii) a DNA hybridizing to the DNA of (i) or (ii) under a stringent condition.

[6] The pathogen-responsive promoter according to any one of [1] to [5], which is characterized by being responsive specifically to Phytophthora infection.

[7] A DNA comprising nucleotide sequence shown in SEQ ID NO:23.

[8] A DNA comprising 10 or more continuous nucleotides of nucleotide sequence shown in SEQ ID NO:23 and having pathogen-responsive promoter activity.

[9] A vector comprising the pathogen-responsive promoter according to any one of [1] to [6].

[10] A vector comprising the DNA according to [7] or [8].

[11] A DNA construct comprising the promoter according to any one of [1] to [6]

and a gene linked under the control of the promoter and expressed in plant to activate protective response of the plant.

[12] A DNA construct comprising the DNA according to [7] or [8], a DNA cooperatively constituting with the DNA a pathogen-responsive promoter, and a gene linked under the control of the constituted pathogen-responsive promoter and expressed in plant to activate protective response of the plant.

[13] The DNA construct according to [11] or [12], wherein the expression product of the gene has the function to activate communication pathway controlling the protective response of the plant.

[14] The DNA construct according to [11] or [12], wherein the expression product of the gene has the function to activate SIPK or WIPK.

[15] The DNA construct according to [11] or [12], wherein the gene encodes a constantly active form of MEK.

[16] A transformant derived from host plant transformed by the DNA construct according to any one of [11] to [15].

[17] The transformant according to [16], wherein the host plant belongs to Solanaceae.

[18] The transformant according to [16], wherein the host plant belongs to Solanum tuberosum.

[19] A method for producing a transgenic plant, comprising the step of:

transforming a host plant with the DNA construct according to any one of [11] to [15].

[20] A method for affording pathogen resistance to a host plant, comprising the step of:

transforming the host plant with the DNA construct according to any one of [11] to [15].

[21] A plant into which a pathogen-responsive promoter according to any one of [1] to [6] has been exogenously introduced.

[22] A plant into which the DNA according to [7] or [8] has been exogenously introduced.

"Pathogen-responsive promoter" in the present invention means a promoter that is responsive to (induced by) pathogen infection. "Promoter" as used herein refers to a functional region under whose control the initiation of transcription of a gene is regulated.

"Exogenously introduced" as used herein means introduction from outside. Accordingly, "exogenously introduced promoter" means a promoter that is introduced from outside of the host cell. For example, even if the host cell natively possesses a promoter identical to that promoter which is introduced, only the latter is referred to as "exogenously introduced promoter" for discrimination purpose, regardless of whether they are identical in construction.

The phrase "comprising DNA" in the present invention is used to encompass "consisting of DNA". For example, "a promoter comprising a given DNA" is considered to include "a promoter consisting of a given DNA".

In the present specification, the abbreviations shall have the meanings as follows; ATP: adenosine 5'-triphosphate, BPB: bromophenol blue, BSA: bovine serum albumin, CBB: coomassie brilliant blue, CTP: cytidine 5'-triphosphate, DEPC: diethylpyrocarbonate, DTT: dithiothreitol, EDTA: ethylenediamine-N, N, N', N'-tetraacetic acid, EGTA: ethyleneglycol bis (ß-amonoethylether) ethylenediamine-tetraacetic acid, FPP: farnesyl diphosphate, GAP: glyceraldehyde 3-phosphate, GTP: guanosine 5'-triphosphate, HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A, HMGR: 3-hydroxy-3-methylglutaryl coenzyme A reductase, HWC: hyphal wall components, Ig: immnoglobulin, IPP: isopentenyl diphosphate, IPTG: isopropyl-1-thio-ß-D-thiogalactoside, kD: kilodalton, MOPS: 3- (N-morpholino) propanesulfonic acid, PAGE: polyacrylamide gel electrophoresis, PBS: phosphate-buffered saline, PCR: polymerase chain reaction, PMSF: phenylmethylsulfonyl fluoride, PR: pathogenesis related, SDS: sodium dodesyl sulfate, SHAM: salycylhydroxamic acid, SSPE: sodium chrolide-sodium phosphate, EDTA, TBE: tris-borate, EDTA, TBS: tris-buffered saline, TE: tris-EDTA, Tris: 2-N-tris (hydroxymethyl) aminomethane, TTP: thiamine 5'-triphosphate, X-gal: 5-bromo-4-chloro-3-indolyl-ß-D-galactoside.

Unless otherwise specified, the genetic engineering operations in the following description can be performed with reference to Molecular Cloning (Third Edition, Cold Spring Harbor Laboratory Press, New York) or Current protocols in molecular biology(edited by Frederick M. Ausubel et al., 1987).

The present invention provides a promoter that is induced by pathogen infection. Gene transfer using the present promoter allows creation of transgenic plant wherein a desired gene is expressed specifically on the occasion of pathogen infection. Accordingly, by transferring any gene involved in protective response, pathogen-resistant plant can be created which evokes a prompt protective response to pathogen infection.

BRIEF DESCRIPTION OF DRAWINGS

Fig.1 A sheme of stimulus-responsive isoprenoid biosynthesis in potato tuber. Wound-induced sterol and steroid glycoalkaloid syntheses are suppressed in favor of sesquiterpenoid phytoalexin synthesis during expression of the hypersensitive response.

Fig. 2 Expression of PVS (potato vetispiradiene synthase) and PSS (potato squalene synthase) genes in aged potato discs after inoculation with *P. infestans*. Potato discs were aged for 24 hr prior to inoculation with 10^4 zoospores per discs of race 0 (incompatible) or of race 1, 2, 3, 4 (compatible), or treatment with water.

Fig. 3 RT-PCR using the total RNA from aged potato discs after inoculation with incompatible (Incomp.) or compatible (Comp.) race of *P. infestans* or, treatment with water (Mock). Clone specific-primers of *PVS1, PVS2, PVS3,* and *PVS4* were used for PCR and will generate products of 469, 132, 326, and 469 bp, respectively.

Fig. 4 Western blot analysis using total proteins from aged potato discs after inoculation with the incompatible (Incomp.) or compatible (Comp.) rave of *P. infestans*, or treatment with water (Mock). Each 10 μg totatl proteins were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted using antiserum against PVS. The detection was carried our using HRP-linked anti-mouse Ig and ECL detection kit.

Fig. 5 RT-PCR using total RNA from potato leaves treated with water (Mock) or after wounding (Wound.), or inoculation with the incompatible (Incomp.) or compatible (Comp.) race of *P. infestans*. Lane TI; RT-PCR products from potato tuber 6 hr after inoculation with the incompatible race of *P. infestans* were loaded as a positive control. Member specific-primers of *PVS1, PVS2, PVS3,* and *PVS4* were used for PCR and will generate products of 176, 132, 326, and 131, respectively. RT-PCR products were separated by agarose gel electrophoresis and blotted onto nylon membranes. Membranes were hybridized with each ^{32}P labeled PCR product.

Fig. 6 shows deduced nucleotide sequence and deduced amino acid sequence

of PVS3 genomic clone, with a portion of deduced promoter and coding regions shown. Amino acid sequences are indicated below the nucleotide sequences by which they are encoded. Non-coding regions are indicated by lower case letters. Stop codons are marked with asterisks.

Fig. 7 shows deduced nucleotide sequence and deduced amino acid sequence of PVS3 genomic clone, with a portion of coding region and untranslated region shown. Amino acid sequences are indicated below the nucleotide sequences by which they are encoded. Non-coding regions are indicated by lower case letters. Stop codons are marked with asterisks.

Fig. 8 Restriction and structural maps for *PVS3* genomic clone and cDNA clones. Coding regions are represented by open boxes with introns shown as thick lines. Vertical bars correspond to intron positions.

Fig. 9 A schematic representation of an amino acid sequence alignment between *N. tabacum* (TEAS) *S. tuberosum* (PVS) *H. muticus* (HVS) *C. annum* (PEAS). Sequence alignments used deduced amino acid sequence corresponding to exons. Solid vertical bars correspond to intron positions within the *N. tabacum, S. tuberosum, H. muticus* and *C. annum* genes. Numbers within the boxes indicate the number of amino acids encorded by exon. Percentages refer to identity scores between the indicated domains, and H, C, and DDXXD (or DDXX) refere to conserved histidine-, cysteine, and aspartate-rich (and known as the substrate binding site) residues.

Fig. 10 Luciferase activity by the treatment with hyphal wall components (HWC) elicitor or water in electroporated potato protoplasts. (A) Construc of Luc gene for transient assay using *PVS3* promoter region. In (B), 35S represents luciferase activity when CaMV 35S promoter region was used, HWC represents that activity when the deduced promoter region was used and HWC treatment was performed, and Water represents that activity when water treatment was performed instead of HWC treatment.

Fig. 11 Schematic representation of the construct of *PVS3* promoter. GUS reporter gene.

Fig. 12 Expression pattern of GUS driven by *PVS3* promoter in response to wounding. Transgenic potato leaves carrying *PVS3* promoter were used.

Fig. 13 Expression pattern of *PVS3* promoter in response to inoculation with *P. infestans*. Transgenic potato leaves (MayQueen) or potato leaves (Rishiri) were inoculated with race 0 (compatible for MayQueen and incompatible for Rishiri). GUS activity was detected by GUS staining solution 6, 12, 24, and 48 hr after

inoculation. Transgenic potato leaves were observed under the microscope.

Fig. 14 Expression pattern of GUS driven by *PVS3* promoter in transgenic potato plants. GUS activity of transgecin potato plants was detected by GUS staining solution solution. Arrows indicate *P. infestans* inoculation areas as GUS staining control.

Fig. 15 Expression pattern of GUS driven by *PVS3* promoter in response to arachidonic acid (AA). AA (5 mM) or water was injected into transgenic potato leaves. GUS activity was detected using GUS staining solution 6, 12, and 24 hr after injection,.

Fig. 16 Expression pattern of GUS driven by *PVS3* promoter in response to H_2O_2. H_2O_2 (5 mM) was injected into transgenic potato leaves. GUS activity was detected using GUS staining solution 6, 12, 24, and 48 hr after injection.

Fig. 17 Expression pattern of GUS driven by *PVS3* promoter in response to glucose/glucose-oxydase. Glucose (5 mM) and glucoseoxydase (0.5 U/ml) were injected into transgenic potato leaves. GUS activity was detected using GUS staining solution 6, 12, 24, and 48 hr after injection.

Fig. 18 Expression pattern of GUS driven by *PVS3* promoter in response to salicylic acid (SA). SA (0.5 U/ml) was injected into transgenic potato leaves. GUS activity was detected using GUS staining solution 6, 12, 24, and 48 hr after injection.

Fig. 19 Expression pattern of GUS driven by *PVS3* promoter in response to Cf-9/Avr9 interaction or $StMEK^{DD}$. *Agrobacterium* carrying 35S:Cf-9/Avr9, $StMEK^{DD}$ or empty vector (control) was infiltrated into transgenic potato leaves. GUS activity was detected using GUS staining solution 2 days after *Agrobacterium* infiltration.

Fig. 20 Schematic representation of elicitor-Induced signal transduction. MAPKKK; mitogen-activated protein kinase kinase kinase, MAPKK; mitogen-activated protein kinase kinase, MAPK; mitogen-activated protein kinase, SIPK; salicylic acid-induced protein kinase, WIPK; wound-induced protein kinase, HMGR; 3-hydroxy-3-methyglutaryl CoA reductase, PVS; potato vetispiradiene synthase.

Fig. 21 shows the sequence of coding region of MEK gene (MEK) of potato plant and deduced amino acid sequence encoded by the MEK gene.

Fig. 22 shows the sequence of coding region of constantly active form of MEK gene ($StMEK^{DD}$) and deduced amino acid sequence encoded by the MEK gene.

Fig. 23 shows the positions of primer sequences used in Examples. Numerals

which are accompanied by arrows pointing at each primer position represent sequence identification numbers (For example, P9 represents a primer having the sequence of SEQ ID NO:9). In Example 2, P9, P10, P11, P14, P15, P16, P17, and P18 were used. On the other hand, in Example 4, P11, P12, P13, P14, P15, P16, P19, and P20 were used.

Fig. 24 shows primer sequences used in construction of deletion clones of pPVS3-1 through pPVS3-10. F denotes a forward primer, R denotes a reverse primer, and underlines indicate positions of restriction enzymes.

Fig. 25 shows the construction of a binary vector including PVS promoter region used in the transient assay. GUS gene includes introns.

Fig. 26 shows the method for examining the PVS3 promoter activity for INF1 treatment or StMEK1^{DD} expression. For INF1 treatment, Agrobacteria containing PVS3 : GUSint was injected to leaves. For StMEK1^{DD} expression, a mixture of Agrobacteria containing PVS3 : GUSint and XVE : StMEK1^{DD} was injected into leaves. The leaves were left stood for one day (A). Subsequently, for INF1 treatment, INF1 solution was injected into leaves. For StMEK1^{DD} expression, ß-estradiol was injected (B) before the leaves were left stood for one day to bring about StMEK1^{DD} expression, and GUS activity determined (C).

Fig. 27 shows a procedure for virus-inducible gene silencing. (A) shows the construction of silencing vector pGR106. Gene fragments to be silenced, cDNA fragments of SIPK and WIPK in this case, are inserted to pGR106. (B) schematically shows the virus infection caused by inoculation with Agrobacterium containing a vector.

Fig. 28 shows PVS3 promoter activity responsive to INF1 treatment. In -1337(pPVS3-2), GUS activity was induced by injecting INF1, in contrast to control area. On the other hand, deletion of PVS3 promoter up to -1,287 (pPVS3-3) significantly reduced GUS activity induced by INF1 treatment.

Fig. 29 shows the nucleotide sequence and deletion position from pPVS3-1 to pPVS3-10 of PVS3 promoter. Sequence between -1,337 and -1,287, where cis-sequence is expected to lie, is shown by bold letters, and deduced TATA box and CAAT box are enclosed in square.

Fig. 30 shows the PVS3 promoter activity in response to StMEK1^{DD}. In -1,337 (pPVS3-2), higher GUS activity compared to control area was induced by injecting ß-estradiol. On the other hand, deletion of PVS3 promoter up to -1,287 (pPVS3-3)significantly reduced GUS activity induced by ß-estradiol.

Fig. 31 shows the method for examining effect of WIPK or SIPK on PVS3 promoter activity induced by $StMEK1^{DD}$ expression. A mixture of Agrobacteria containing PVS3:GUSint and XVE:$StMEK1^{DD}$ was injected into silencing leaves and left stood for one day (A). ß-estradiol was then injected (B) and the leaves left stood again for another one day to allow $StMEK1^{DD}$ expression. The resultant GUS activity was examined (C).

Fig. 32 shows the effect of WIPK and SIPK on PVS3 promoter activity and TEAS gene expression induced by $StMEK1^{DD}$ expression. Silencing of either WIPK or SIPK significantly suppressed the PVS3 promoter activity induced by $StMEK1^{DD}$ (A). Further, Northern analysis of the total RNA extracted demonstrated that the expression of sesquiterpene cyclase gene of Nicotiana benthamiana was suppressed only at the area where WIPK and SIPK were silenced (B).

BEST MODE FOR CARRYING OUT THE INVENTION

(Promoter)

A first aspect of the present invention relates to a pathogen-responsive promoter, an embodiment of which comprises DNA including the nucleotide sequence shown in SEQ ID NO:1. The DNA has been identified as promoter of potato PVS3 gene, and has been recognized as being responsive specifically to Phytophthora infestans, a type of virulent pathogen. As a result of further examination, it was found that deletion of upstream region resulting in a fragment consisting of as few as about 1,300 bp (SEQ ID NO:22) retained the intended promoter activity. In view of these findings, a preferred embodiment of the present invention is a pathogen-specific promoter comprising DNA having the nucleotide sequence shown in SEQ ID NO: 22. On the other hand, deletion of a region of 50 bp (SEQ ID NO: 23) upstream the DNA sequence (SEQ ID NO:22) resulted in a dramatic decrease of the promoter activity. Thus, the deleted 50bp (SEQ ID NO:23) is expected to comprise an extremely essential region for the promoter activity, i.e. cis-sequence of PVS3 gene promoter. Accordingly, the region (hereinafter referred also to as "first DNA sequence") is highly useful for construction of a pathogen-responsive promoter, and the use of the region allows flexible design and construction of DNA construct incorporating the pathogen-responsive promoter (for example, recombinant vector for use in imparting pathogen resistance to plant. See the section of "DNA construct" below.). Thus, another embodiment of the present invention provides DNA sequence useful for

construction of a pathogen-responsive promoter. Since cis-sequences in general consist of ten and several nucleotides, a portion of the first DNA sequence is predicted to be a cis-sequence. This implies that DNA consisting only of a portion of first DNA sequence may be useful for construction of a pathogen-responsive promoter as long as it contains a cis-sequence. An exemplary DNA encompassed in such a DNA comprises continuous 10 or more nucleotides, preferably continuous 15 or more nucleotides, and more preferably continuous 20 or more nucleotides of the first DNA sequence.

When the first DNA sequence (or a continuous portion thereof, or those DNA (Modified DNA) obtained by modifying the first DNA sequence or a continuous portion thereof as specified below such that its function will be retained) is used, a pathogen-responsive promoter can be constructed by incorporating any other DNA sequences into the first DNA sequence. The other DNA sequences herein referred to are those which cooperate with the first DNA sequence (or its Modified sequence) to construct a pathogen-responsive promoter. Specifically, the sequence shown in SEQ ID NO:24, for example, may be used as the other DNA sequence. The DNA sequence is the DNA sequence of that region which is flanked by the first DNA sequence and the coding region of PVS3 gene (See Fig. 29). Since this flanked region includes CAAT box and TATA box, and a native PVS3 promoter region is constructed by using this flanked region, the pathogen-responsive promoter created in this embodiment will have a high promoter activity. In addition to this example, any other DNA sequence including CAAT box and TATA box is expected to provide a favorable promoter function through cooperation with these sequences involved in transcription initiation and transcription regulation. The other DNA sequence may be linked directly, or via any other sequence, with the first DNA sequence.

The term "pathogen" as used herein refers to any fungus that infects to and harms plants, including Phytophthora and other virulent filamentous fungi and virulent bacteria. "Phytophthora" as used herein refers to any fungi that belongs to the genera of Phytophthora, and is classified according to infected subject. Exemplary Phtophthora are: potato Phytophthora (Phytophthora infestans), tobacco Phytophthora (Phytophthora nicotianae), soybean pedicle Phytophthora (Phytophthora megasperma var. sojae), and apple Phytophthora (Phytophthora cactorum and Phytophthora cambivora). Exemplary virulent filamentous fungi are: potato Sclerotium

(Sclerotinia sclerotiorum), Pyricularia oryzae (Magnaporthe grisea), and soybean rust (Phakopsora pachyrhizi). Exemplary virulent bacterium is fungi of bacterial disease of tomato (Ralstonia solanacearum, bacterium).

The promoter according to the present invention (comprising DNA useful for construction of a pathogen-responsive promoter. Unless otherwise indicated, this definition holds hereinafter) preferably is responsive specifically to pathogen infection. The term "specifically" means having a high specificity. Accordingly, the promoter according to the present invention preferably has a high specificity to pathogen infection, i.e. is responsive to pathogen infection as well as substantially lacks responsiveness to those disease other than pathogen infection.

(Method for Obtaining the Promoter)

The promoter according to the present invention can be prepared from potato plant such as Solanum tuberosum L. by extracting its genomic DNA using any standard technique, and then performing PCR or other gene amplification reaction using primers specific to the promoter of the present invention. Specifically, the promoter according to the present invention can be prepared in accordance with the following procedures. First, harvested and frozen potato leaves or tubers are ground in a mortar. Subsequently, an appropriate amount of extraction buffer (Tris-HCl buffer containing SDS, for example) is added to obtain extraction, followed by extraction and purification of genomic DNA by phenol extraction, ethanol precipitation or other means. The genomic DNA thus obtained is used as a template to perform PCR using primers specific to the promoter shown in SEQ ID NO:1 to obtain DNA (promoter) of interest as amplification product. Primers used may be, for example, a pair of primers having the following sequences:

Sense primer : TTGTCTGCTGCTGCTTGTGG(SEQ ID NO: 15)

Antisense primer: TCTCCATGAGTCCTTACATG(SEQ ID NO:16)

Primers are designed to amplify specific DNA of interest. A set of primers for specifically amplifying DNA shown in SEQ ID NO:22 are:

Sense primer: CGGAATTCGTCCGCCCTTACTATTCCCATC (SEQ ID NO:26)

Antisense primer: CCATCGATTCCTCTTCATTGTTAAAGGGGA(SEQ ID NO:35)

Method for preparing the promoter according to the present invention is not limited to that described above. For example, commercially available potato genomic library (for example, genomic library of potato race Desiree (Clontech))may be used for preparation. In order to isolate the promoter of interest from such a genomic library, plaque hybridization or colony hybridization, among others, may be used depending on the type of library (See Molecular Cloning, Third Edition, Cold Spring Harbor Laboratory Press, New York, for example). For example, in the case of library constructed using phage, plaque hybridization may be used. Selection of clone having the promoter region of interest may be achieved by using any probe having a sequence specific for the promoter of the present invention.

Once the clone of interest has been selected, the DNA contained in the clone may be used as template of PCR or other reaction using primers specific to the sequence shown in SEQ ID NO:1 to obtain the promoter of the present invention as amplification product.

The DNA contained in the clone obtained can be subcloned into any appropriate vector for further use. This allows construction of recombinant vector useful for transformation (See the second aspect of the present invention that will be described later) or construction of plasmid that is useful for sequencing of nucleotides.

Method for preparing the promoter according to the present invention is not limited to those described above, and any commercially available DNA synthesizer, for example, may be used for synthesis of the present promoter.

(Modified Promoter)

Since the sequence shown in SEQ ID NO:1 is about 2,600 bp long and relatively huge as promoter region, those nucleotides which are effectively involved in promoter activity is expected to be only a portion thereof. In view of this, even a partial continuous region of the sequence shown in SEQ ID NO:1 may constitute a pathogen-responsive promoter according to the present invention as long as it proves to function as a pathogen-responsive promoter. In addition, functional region of a promoter is usually located immediately before its structural gene. Therefore, in the case of sequence of PVS3 gene shown in Figs. 6 and 7, the region consisting of -2,000

through -1 (SEQ ID NO:2), preferably the region consisting of -1,500 through -1 (SEQ ID NO:3), more preferably the region consisting of -1,000 through -1 (SEQ ID NO:4) may be promising candidates for functional region. As a matter of fact, as indicated in examples below, it has been demonstrated that the region which is about 1,300 bp in length immediately before the structural gene (SEQ ID NO:22) has a promoter activity. On the other hand, deletion of a region of 50 bp (SEQ ID NO:23) further upstream the approximately 1,300 bp region resulted in a dramatic decrease of promoter activity. Thus, it was found that at least one of functional regions of PVS3 promoter lies within this region of 50 bp. Based on this fact, the promoter according to the present invention preferably includes the sequence shown in SEQ ID NO:23. One example of such a promoter is DNA shown in SEQ ID NO:22 (-1,337 through -1 of PVS3 gene).

Meanwhile, DNA may occasionally retain its function even when partially modified. In view of this, any DNA having a partially modified nucleotide sequence (also referred to as "modified DNA") compared to the above described DNA (namely, DNA shown in SEQ ID NO:1, or DNA (for example, DNA shown in SEQ ID NO:22) including the above described functional region (SEQ ID NO:23)) constituting the promoter according to the present invention can constitute a pathogen-responsive promoter as long as it retains the function as pathogen-responsive promoter. In other words, partial modification of the sequence is permissible as long as its pathogen-responsive promoter function is retained. The term "partial modification" as used herein refers typically to replacement, deletion, insertion, or addition of one or more nucleotide(s) in the sequence shown in SEQ ID NO:1 (any one of SEQ ID Nos:2 through 4) or in the sequence shown in SEQ ID NO:22. Such modification can be made on a plurality of locations. The term "plurality" as used herein varies, depending on the site or type of modification made, for example between 2 and 100, preferably 2 and 50, more preferably 2 and 10. Such modified DNA can be achieved by inserting mutation by, for example, treatment by restriction enzyme, treatment by exonuclease, DNA ligase or other enzyme, site-directed mutagenesis (Molecular Cloning, Third Edition, Chapter 13, Cold Spring Harbor Laboratory Press, New York), and random mutagenesis (Molecular Cloning, Third Edition, Chapter 13, Cold Spring Harbor Laboratory Press, New York).

Any DNA which hybridizes under a stringent condition with DNA comprising

the sequence of SEQ ID NO:1 (or any one of SEQ ID Nos:2 through 4), or with DNA comprising the sequence of SEQ ID NO:22 and also functions as pathogen-responsive promoter in plant cell can be used as DNA constituting the promoter according to the present invention. Further, any DNA which hybridizes under a stringent condition with DNA comprising the sequence with the above partial modification on the sequence of SEQ ID NO:1 (or the sequence with the above partial modification on the sequence of SEQ ID Nos:2 through 4), or with DNA comprising the sequence with the above partial modification on the SEQ ID NO: 22 and also functions as pathogen-responsive promoter in plant cell can be used. The term "stringent condition" as used herein refers to those conditions under which a so-called specific hybrid is formed, and non-specific hybrid not formed. Stringent conditions vary depending on the length of sequence and the type of constituent nucleotides. Exemplary condition is incubation in hybridization solution (50% formaldehyde, 10 × SSC (0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 5 × Denhardt solution, 1% SDS, 10% dextran solution, 10 μg/ml of denatured salmon sperm DNA, 50mM phosphate buffer (pH 7.5)) at 42°C, followed by washes in 0.1 × SSC, 0.1% SDS at 68°C. An example of more preferable stringent conditions is such a condition using a solution of 50% formaldehyde, 5×SSC (0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 1 × Denhardt solution, 1% SDS, 10% dexstran sulfate buffer, and 10 μg/ml of denatured salmon sperm DNA in 50 mM phosphate buffer (pH 7.5) as hybridization solution.

(DNA Construct)

By linking, under the control of the promoter of the present invention, any gene (transgene) which activates, upon expression, protective responsive of plant, DNA construct can be produced which is useful in imparting pathogen resistance to the plant. When producing a DNA construct for use in transformation, it is preferred that the promoter of the present invention and transgene of interest are incorporated into any appropriate vector (plasmid, bacteriophage, or virus, for example).

(Transgene)

Any transgene is used which activates protective response of the plant into which it has been introduced. For example, any gene that activates a communication pathway controlling protective response of the plant may be used as transgene. Exemplary genes of this type are MEK genes which activate SIPK (salicylic acid-induced protein kinase), a mitogen-activated protein (MAP) kinase, or WIPK

(Wound-Induced Protein Kinasae). An example of MEK genes is shown in Fig. 21 as the sequence of coding region of potato MEK gene (StMEK)(SEQ ID NO:5) and the amino acid sequence (SEQ ID NO: 6) encoded thereby.

It is especially preferable to use as transgene any gene encoding a constitutive active form of protein, because such a gene can produce an originally active form of protein that activates and effects protective response of the transformant in a prompt and assured manner. Genes encoding constitutive active form of proteins can be produced by partially modifying the nucleotide sequence of any gene encoding a wild type of protein such that the encoded amino acid sequence is partially mutated. For potato plant, a constitutive active form of protein MEK ($StMEK^{DD}$) having a modified MEK has been produced, and the corresponding gene encoding the $StMEK^{DD}$ may be used in the present invention as transgene. The nucleotide sequence of the coding region of $StMEK^{DD}$ gene (SEQ ID NO:7) and the encoded amino acid sequence (SEQ ID NO:8) are shown in Fig. 22.

The type of "protective response" in the above description is not limited in any way, and encompasses production of phytoalexin, expression of PR (Pathogenesis-Related) protein, production of reactive oxygen species, formation of papilla, and lignification.

(Vector)

Vectors for use in production of the above-described DNA construct are not limited, as long as they can introduce the promoter of the present invention and a transgene to be placed under control thereof into a target cell (host cell) and allow expression of the transgene within the target cell. Depending on the purpose, plasmid vector and λ phage vector, for example, are used. In constructing a vector for use in transformation using Agrobacterium as will be described later, Ti plasmid binary vector, Ti plasmid vector having T-DNA boundary sequence, for example, can be used. When the vector is used for any transformation without the need of Agrobacterium (for example, electroporation, and particle gun), a variety of pUC series plasmid vectors, a variety of λ phage vectors (ZAPII and others) may be used to construct a recombinant vector. A number of vectors are commercially available, an appropriate one among which may be selected, depending on the purpose, for use in the present invention.

A vector that contains the promoter of the present invention may first be constructed, and then a transgene be linked thereto. Namely, a universal vector that permits insertion of a desired transgene may first be constructed, and then be used for production of a recombinant vector for performing transformation.

The vector for performing transformation typically contains a transgene and any appropriate terminator in addition to the promoter of the present invention. The promoter, transgene and terminator are positioned in this order toward downstream such that transcription of the transgene by the promoter can be appropriately effected. The recombinant vector may further contain a selectable marker, a sequence having an enhancer function, and a sequence encoding any signal peptide.

(Terminator)

Terminator is a sequence that is recognized as signal to terminate synthesis of mRNA. Any terminator that functions correctly in plant cell is used. For example, Nos terminator is useful.

(Selectable Marker)

Selectable marker is used for recognizing or selecting those cell, tissue or callus that has underwent transformation. A variety of selectable marker are known, including, for example, npt gene (Herrera Estrella, EMBO J. 2(1983), 987-995) and nptII gene (Messing & Vierra. Gene 1 9:259-268 (1982)) for imparting resistance against kanamycin, hph gene (Blochinger & Diggl mann, Mol Cell Bio 4:2929-2931) for imparting resistance against hygromycin, dhfr gene (Bourouis et al., EMBO J. 2(7)) for imparting resistance against methotrexate, ß-glucronidase (GUS) gene, GFP gene (Gerdes, FEBS Lett. 389 (1996), 44-47), luciferase (Giacomin, P1. Sci. 116 (1996), 59 to 72; Scikantha, J. Bact. 178 (1996), 121) and may be selected for use depending on the vector's host system and application.

The promoter and transgene may be inserted to the vectors by any standard technique, such as by using restriction enzyme DNA ligase (See Molecular Cloning, Third Edition, 1.84, Cold Spring Harbor Laboratory Press, New York, for example).

(Method for Transformation)

The DNA construct or recombinant vector according to the present invention

can be used for transformation of plants. Transformation (gene transfer) can be achieved, for example, by means of Agrobacterium tumefaciens (Agrobacterium Method), polyethylene glycol (polyethylene glycol Method), and particle gun wherein metal particles with gene of interest bound thereto are shot into plant tissue (cell). Details of each of these methods are described in a variety of references and publications. For Agrobacterium Method, for example, see Proc. Natl. Acad. Sci. USA 86 (1989), 8467-8471 and Plant Mol. Biol. 20 (1992), 963-976.

Transformation of potato plant can be achieved by the method according to Jefferson (1987) (See Reference 19).

In the transformant obtained through the DNA construct containing the promoter of the present invention and any transgene, the introduced promoter can be induced in response to pathogen infection, and the transgene which is under the control thereof expressed. Accordingly, any transgene that is effective in providing protection against pathogen infection can be used to obtain a plant (transformant) which is resistant to pathogen. A plant cell obtained through the transformation can be used to regenerate a transgenic plant. Such regeneration can be achieved by any standard technique, depending on the type of plant.

(Target Plant)

The plant to be transformed by means of the DNA construct or recombinant vector according to the present invention (target plant) is not limited but may be any dicotyledon or monocotyledon. Dicotyledon includes, for example, Solanaceae (Solanum tuberosum, tobaccos, tomatoes), Osmandaceae (Prunus mume, Prunus persica, Mulus pumila), Leguminosae (for example, Glycine max, Pisum sativum), Brassicaceae (for example, Raphanus sativus), Pedaliaceae. Monocotyledon includes, for example, Gramineae (Oryza sativa, Triticum, cereale, Hordeum vulgare, Coix lacryma-joli, maize, Saccharum, for example), Liliaceae (Allium fistulosum, Allium sativum, for example).

The present invention will be described in greater detail with reference to the following Examples.

[Examples]

Biological materials, reagents, experimental procedures and other details employed in the Examples 1 through 8 below are as follows.

1. Tested plants

Cultivar Danshaku (Solanum tuberosum L.) having no true resistance gene R1 and cultivar Rishiri (interspecies hybrid between Solanum tuberosum L. and wild species S. demissum L.) having a true resistance gene R1 were used as potato plant. Tubers of Danshaku cultured at the farm of Department of Agricultural Science, Nagoya University, and harvested in July, and tubers of the Rishiri cultured at the field of Hokkaido National Agricultural Experimental Station, the Ministry of Agriculture, Forestry and Fisheries and harvested in October were kept at 4°C before testing. For producing a transgenic plant, MayQueen having no true resistance gene was used.

2. Tested Pathogen

Potato Phytophthora, [Phytophthora infestans (Mont.) de Bary] race 0 and races 1, 2, 3, and 4 stored in the Laboratory of Bioresource functions, Graduate School of Bioagricultural Sciences, Nagoya University were used. In addition, as a pathogen for use in preparing potato Phytophthora body wall component (HWC) elicitor, potato Phytophthora [Phytophthora infestans (Mont.) de Bary] races, 1, 2, 3 and 4 stored in the same laboratory was used.

3. Preparation of Pathogen Inoculum

Planospore suspension of potato Phytophthora was prepared as follows. Tubers of potato (Solanum tuberosum L.) that had been stored at 4°C was washed extensively with tap water, and immersed in 1% sodium hypochlorite for about 10 minutes. The tubers were then sliced (into the thickness of about 10 mm) , washed with water, had the planospore suspension that had been prepared in about 10^4 zoospore/ml applied thereon for inoculation, and cultured for six days at 20°C under a humidified darkness. Pathogens that grew out on the slice surface was stripped off with forceps, and suspended in cold distilled water (4°C). The spore suspension was filtered through metal mesh (356 gauge) to remove fungal threads, and suctioned filtered using a paper filter (ADVANTEC No. 5B). The zoosporangia collected on paper filter was washed with cold distilled water, suspended again in cold distilled water, and left stood at 10°C for two hours. The planospore suspension was explored for its absorbance using ultraviolet visible light spectroscopy analysis system (DU

series 600, Beckman), and its concentration adjusted such that its absorbance at wavelength 500 nm becomes 0.068 (10^5 zoospores/ml) before use as inoculum.

4. Preparation of Body Component Elicitor

According to the method of Doke and Tomiyama, (1980) (See Reference 14), hyphal wall components (HWC) of Phytophthora infestans were prepared as follows. Mycelia of Phytophthora were placed in standing culture in 100 ml conical flask containing 30 ml of rye medium at 20°C for two weeks. Mycelial mat collected was washed with tap water, had its humidity removed by suction filtration, and cryopreserved at -80°C. The frozen mycelia were milled in mortar, and suspended in 50 mM of acetate buffer (pH 4.5) of 5 times the weight of mycelia. The suspension was sonicated for five minutes using Sonicator (W-225R Heat System-Ultrasonics Inc.) at 45 W output, and centrifuged at 14,000 x g for 30 minutes. The resultant precipitate was suspended in 50 mM acetate buffer (pH 4.5) of the weight equivalent to that in the previous step, and supersonication and centrifugation performed under conditions again similar to the previous step. The precipitate thus obtained was suspended in 0.1 M borate buffer (pH 8.8) of the weight equivalent to the original weight of mycelia, sonicated under the similar condition to the above, autoclaved at 120°C for 20 minutes, and centrifuged at 14,000 x g for 30 minutes to collect the supernatant. On the other hand, its precipitates was again suspended in 0.1 M borate buffer (pH 8.8), sonicated and autoclaved before centrifugation. Supernatant obtained by the centrifugation was combined with the previous supernatant, and dialyzed through dialysis tube (exclusion limit molecular weight; 12,000) against water at 4°C for 24 hours. The solution after the dialysis was combined with an equivalent volume of diethylether using a separatory funnel, and left stood. Emulsion layer was collected, and its ether was vacuum desiccated using evaporator. The resultant concentrate was added an appropriate amount of water before lyophilization. The lyophilized preparation thus obtained was used as HWC in the following experiments. The HWC was sonicated using a sonicator at 45W output for three minutes and suspended in water before use.

5. Preparation of Potato Tuber Disc

Potato tuber discs were prepared as follows. Potato tubers (Cultivar Rishiri) that had been stored at 4°C were washed extensively with tap water, and immersed in 1% sodium hypochlorite for 10 minutes. The tubers were bored out with a cork borer

(20 mm in diameter) in axial direction to produce columnar pieces of soft tissue. The columnar pieces were then sliced by microtome into discs of 2 mm thickness. The discs thus prepared were washed with cold distilled water (about 4°C) , aligned in a plastic chamber, left stood under humidified darkness, and aged for 21 hours. All of these procedures were conducted under darkness.

6. HWC Treatment and Pathogen Inoculation

The potato tuber discs prepared according to the method described in the above section 5 were pretreated with 100 μl each of distilled water, and left stood for three hours. The discs were then treated with 1 mg/ml HWC, or with 100 μl distilled water as control. When inoculating the discs with potato Phytophthora, 100 μl each of suspension (10^5 spores/ml) was inoculated while rotating in order to obtain a uniform density of planospore. When inoculating potato leaves with the pathogen, each of the leaves was pretreated with 500 μl of distilled water, and left stood for three hours before 500 μl each of suspension was inoculated while rotating in order to obtain a uniform density of planospore on each leaf tissue.

The discs and leaf tissues after the treatment and inoculation were left stood at 20°C under humidified darkness for a selected time period. After this treatment, a set consisting of three pieces of the potato tuber discs and eight leaf tissues were wrapped in an aluminum foil, placed in liquid nitrogen for cryopexy, and stored at -80°C.

7. Extraction of Total RNA from Potato Leaves or Tubers

Extraction of total RNA was performed according to the method described in Yoshioka et al. (1996) (See Reference 52). 2 g each of potato leaves or tuber discs were milled while being added liquid nitrogen in mortar, put into a sterilized centrifuge tube that had been treated with DEPC and contained 5 ml of extraction buffer [100 mM Tris-HCl (pH 9.0), 100 mM NaCl, 1 % SDS], 1 ml of 2-mercaptoethanol, 2.5 ml of 1 M Tris (pH 9.0) saturated phenol, 2.5 ml of chloroform isoamylalcohol (24:1; v/v), thoroughly suspended, and centrifuged (8,000 rpm, 15 minutes). To the collected supernatant, one twentieth amount of 5 M sodium chloride, 5 ml isopropanol were added, and the mixture left stood at -20°C for one hour. Centrifugation (8,000 rpm, 15 minutes) of the mixture produced precipitates, to which 5 ml guanidium salts buffer [4 M guanidine thiothianate, 25 mM sodium acetate (pH 7.0), 0.5% N-lauroyl sarcosine, 20 mM 2-mercaptoethanol], 500 μl 2M sodium

acetate (pH 4.0), 5 ml water-saturated phenol, 1 ml chloroform/isoamylalcohol (49:1, v/v) were added and thoroughly suspended, and the mixture centrifuged (8,000 rpm, 15 minutes). 5 ml isopropanol was added to the supernatant collected, and the mixture left stood at -20°C for one hour. Precipitates produced by centrifugation (8,000 rpm, 15 minutes) of the mixture was suspended in 300 μl guanidium salts buffer, a equivalent amount of isopropanol added, and the mixture left stood at -20°C for one hour. Centrifugation (12,000 rpm, 15 minutes) produced precipitates, which was washed with 500 μl 3M sodium acetate (pH 5.2) at room temperature, and centrifuged (12,000 rpm, 15 minutes). This washing step was repeated twice. Further, the mixture was washed with 500 μl of 70% ethanol, and centrifuged (15,000 rpm, 15 minutes). Precipitates thus obtained was dissolved in 100 μl of DEPC treated water to provide a total RNA sample.

8. Northern Analysis

The total RNA was fractionated by formaldehyde agarose gel electrophoresis (See Reference 37), and then transferred to and immobilized on Hybond-N^+ nylon membrane (Amersham) using alkaline blotting (See Reference 36). As a probe, leaf PVS1 cDNA was used.

Nylon membrane with RNAs adsorbed thereon was left stood in prehybridization solution [50% formamide, 5 x Denhartz solution (See Reference 37), 5 x SSPE (See Reference 37), 0.5% SDS, 100 μg/ml heat-denatured salmon sperm DNA (Pharmacia)] at 42°C for over one hour. ^{32}P labeled DNA probe was added and hybridized at 42°C for over 16 hours. The membrane was washed sequentially in 0.1% SDS-containing 4 x SSPE at room temperature for 15 minutes (twice), in 0.1% SDS-containing 4 x SSPE at 60°C for 15 minutes, and then in 0.1% SDS-containing 2 x SSPE at 60°C for 15 minutes (once). Autoradiography was performed using X-rays film OMAT-AR (Kodak) and intensifying screen Lighting Plus (Dupont) at -80°C.

9. RT-PCR

RT-PCR was performed using RT-PCR high-Plus (TOYOBO). For cDNA synthesis, 1.0 μg of total RNA, 10 pmol/μl of antisense primers and 10 pmol/μl of antisense primers were used to perform 25 cycles of amplification reaction at 94°C (one minute), 47°C (one minute). The primers used were as follows, with the site for annealing onto corresponding cDNAs shown in Fig. 23.

PVS1: 5'-AGGAGATTGTTCGCCCCATA-3'(SEQ ID NO:9) and 5'-TCTCCATGAGTCCTTACATG-3'(SEQ ID NO:10)(469 bp), or 5'-CATCGATTGTTTTGTACATCTG-3'(SEQ ID NO:11)and 5'-AATAATGATACAAAAAAAAATTAAGG-3'(SEQ ID NO:12)(176 bp)

PVS2: 5'-TATCAATTCACCAAGGAACACT-3'(SEQ ID NO:13)and 5'-GAAGTAATTAAATTTAAATATTATCAA-3'(SEQ ID NO:14)(132 bp)

PVS3: 5'-TTGTCTGCTGCTGCTTGTGG-3'(SEQ ID NO:15)and 5'-TCTCCATGAGTCCTTACATG-3'(SEQ ID NO:16)(326 bp)

PVS4: 5'-AGGACATTGTTCGACCTGTT-3'(SEQ ID NO:17)and 5'-TCTCCATGAGTCCTTACATG-3'(SEQ ID NO:18)(469 bp), or 5'-CATCCCTTAAAATTATAAGTATTC-3'(SEQ ID NO:19)and 5'-AATAATGATACAAAATAAATTAAGG-3'(SEQ ID NO:20)(131 bp)

The cDNAs thus synthesized were fractionated by 2% agarose gel electrophoresis and stained with ethidium bromide to determine the presence or absence of bands (See Reference 37).

10. Preparation of Soluble Fractions from Potato Tuber

Soluble fractions were prepared from potato tuber discs according to the partially modified method of Dixon and Fuller, (1978) (See Reference 13).

A set of three pieces of potato tuber disc was wrapped in an aluminum foil, and stored at -80°C in cryopexy in liquid nitrogen. The frozen potato tuber discs were milled with pestle while being added liquid nitrogen. To the potato tuber powder thus obtained, 2g of polycra AT, a phenol absorbant, was added, and agitated with pestle. Subsequently, 7 ml of extraction buffer [0.1 M sodium borate (pH 8.8) 1 mM PMSF (phenylmethylsulfonyl fluoride), 10 mM 2-mercaptoethanol] was added to bring the mixture into suspension, and cool-centrifuged (14,000 rpm, 20 minutes, 4°C). The resultant supernatant was stored at -80°C.

11. Expression of Fusion Protein in E. coli and its Extraction

In order to obtain antigen for use in antibody production, potato PVS was expressed in E. coli. Full length of translatable region of PVS1 cDNA was inserted into expression vector pET-32b(+)(Takara Shuzo)that had been cleaved with EcoRI and XhoI, and the resultant vector was introduced into E. coli. (BL21, Novagen).

The E. coli was then plated on LB agar medium containing 50 μg/ml carvenicilin, and cultured at 37°C overnight. 50 mL of LB liquid medium with 200 μg/ml carvenicilin was poured into four 500 ml flasks, into which single colonies werestripped off and suspended. The flasks were shake-cultured (140 rpm) at 37°C until A_{600} = 0.6. 250 μl of the sample in the flasks was used as pre-induction protein sample to confirm the expression of fusion proteins. Subsequently, IPTG was added to the final concentration of 1 mM to induce the expression of proteins, and shake-cultured at 37°C (140 rpm) for three hours. After cooling on ice for 5 minutes, the medium was centrifuged (5,000 rpm, 10 minutes). Supernatant was removed, and precipitates was resuspended in 5 ml E. coli suspension [50 mM Tris-HCl (pH 8.0), 2 mM EDTA], and 100 μl of which was used as post-induction protein sample to confirm the induced expression of fusion proteins. The medium was again centrifuged (5,000 rpm, 10 minutes), supernatant removed, and precipitates of E. coli was used to confirm the solubility of fusion proteins.

Confirmation of induced expression of fusion proteins as well as confirmation of solubility was conducted as follows. The pre-induction and post-induction protein samples that have been sampled in the above manner were centrifuged (5,000 rpm, 30 seconds) to remove supernatant, and precipitates resuspended in 100 μl of E. coli suspension. 10 μl each of each suspension was sampled to perform SDS-PAGE and Western analysis. SDS-PAGE and Western analysis were performed according to the section 14. "SDS-PAGE and Western analysis". After confirming the induced expression, E. coli precipitates having the expression of fusion proteins induced was suspended thoroughly in ice-cooled 5 ml Binding Buffer [5 mM imidazole, 0.5 M sodium chloride, 20 mM Tris-HCl(pH 7.9)]. The suspension was transferred into transparent centrifuge tubes, which were ice-cooled while the E. coli inside was being broken by ultrasonicator. The suspension was centrifuged (12,000 rpm, 10 minutes), and supernatant provided as soluble fraction. To the precipitates, 5 ml urea containing Binding Buffer (6 M urea plus Binding Buffer) was added, resuspended, the suspension centrifuged (12,000 rpm, 10 minutes), and supernatant obtained as urea fraction. After 10 μl each of soluble fraction and urea fraction were sampled, SDS-PAGE and Western analysis were performed. The SDS-PAGE and Western analysis were performed according to the section 14. "SDS-PAGE and Western analysis".

Since the fusion proteins were found in the urea fraction, the urea was gradually removed to regenerate the structure of produced protein. Production of proteins was conducted as follows. The urea fraction was transferred into dialysis tubes, and dialyzed against 200 ml of 4 M urea dialysate [4 M urea, 10 mM Tris-HCl (pH 7.0), 5 mM DTT] at 4°C for one hour. The dialysate was exchanged to 200 ml of 2 M urea dialysate (4 M urea dialysate with its urea concentration changed to 2 M), and dialyzed at 4°C for one hour. The dialysate was further exchanged to 200 ml of urea-free dialysate (4 M urea dialysate with its urea removed) and dialyzed at 4°C for one hour. The dialysate was again exchanged to 200 ml of urea-free dialysate (4 M urea dialysate with its urea removed), and dialyzed at 4°C overnight. The dialysed solution was transferred into Eppendorf tubes, and centrifuged (15,000 rpm, 10 minutes), and supernatant transferred into new tubes. This fraction served as regenerated fraction, and was used as antigen for production of antibody. As a result of such procedures, 4 ml of 8 mg/ml fusion proteins was obtained.

12. Production of Anti-PVS antibody

Mouse (BALB/c, female, 4-week-old) was bred for five days, and injected peritoneally with 100 μl of emulsion containing 100 μg solution of fusion protein expressed in E. coli admixed with an equivalent amount of Complete Freund Adjuvant (DIFCO). One week later, 100 μl of emulsion containing the 100 μg fusion protein admixed with an equivalent amount of Incomplete Freund Adjuvant (DIFCO) was peritoneally injected. 10 days later, the mouse was bled at their tail, and explored by Western Analysis for production of antibody against HMGR. Since an antigen-antibody reaction was revealed, 100 μl of emulsion containing 100 μg fusion protein admixed with an equivalent amount of Incomplete Freund Adjuvant (DIFCO) was again peritonealy injected. One week later, blood was collected and left stood at 4°C overnight to have blood clot precipitated. The blood was centrifuged (10,000 rpm, 15 minutes), small amount of supernatant aliquoted as antisera into Eppendorf tubes, and stored at -80°C.

13. Quantification of Protein

Concentration of proteins in samples were quantified according to the method of Bradford (1976) using protein quantification kit (BIO-RAD). Calibration curves were generated using BSA.

14. SDS-PAGE and Western Analysis

SDS-PAGE on protein samples were performed according to the method described in Laemmli (1970). 10 μl of sample was mixed with 10 μl of sample buffer [50 mM Tris-HCl (pH 8.5) containing 2% SDS, 10% mercaptoethanol, 0.002% BPB, 20% glycerol], boiled for five minutes, ice-cooled, and run on 10% polyacrylamide gel for electrophoresis.

Western analysis was performed according to the method described in Towbin et al. (1979)(See Reference 55). Gel after SDS-PAGE, paper filter, and nitrocellulose membrane (PROTPRAN, Schleicher and Schuell) were immersed in transfer buffer (0.1 M Tris, 0.192 M glycine, 20% methanol, 0.1% SDS), respectively, for 30 minutes, placed on a stage of semi-dry blotter (ATTO) and driven with 2 mA/cm^2 of constant current for 60 minutes to transfer the protein in the gel onto nitrocellulose membranes. The nitrocellulose membranes were shaken in TBS-T [50 mM Tris-HCl buffer (pH 7.6) containing 137 mM sodium, 0.1% Tween 20] containing 5% skimmed milk overnight, for blocking. The membranes were then washed in TBS-T for 15 minutes once, and for 5 minutes twice, and shaken in TBS-T containing as primary antibody anti-potato PVS antibody (2,000 times dilution) for one hour. The membranes were again washed in TBS-T, and shaked in TBS-T containing anti-mouse-Ig antibody (Amersham) as secondary antibody for 30 minutes. The membranes were washed in TBS-T, and then signals detected using ECL detection kit (Amersham) on Hyper Film (Amersham).

15. Production of Probes

Using plasmids containing introduced potato PVS1 to 4 cDNA as templates, the primers shown in Fig. 23 were used to amplify them by PCR that occurs specifically for nucleotide sequence of each of the genes. The reaction was performed by using 2 ng plasmids having TaKaRa TaqTM (Takara Shuzo) and insert DNA integrated, on DNA thermal cycler PJ2000 (Perkin Elmer Cetus) in 25 cycles of at 94°C for one minute (heat denaturation), at 53°C for 45 minutes (annealing), and at 72°C for two minutes (DNA elongation). The sizes of DNA fragments amplified were examined on 0.8% agarose gel electrophoresis. The DNA fragments were purified from the gel using QIAquick Gel Extraction Kit (QIAGEN).

16. Screening of Potato Genomic Library

As genomic library, commercially available potato genomic library (potato cv. Desiree, Clontech) was used.

Phage clones were selected using plaque hybridization (See Reference 37). Phage solution that had been adjusted to contain 30,000 plaques per plate was mixed with 200 μl of host E. coli XL1-Blue MRA (P2) strain 10 mM magnesium sulfate, A_{600} = 2), left stood at 37°C for 20 minutes, mixed with 3 ml NZYM top-agarose (1% NZ amine, 0.5% yeast extract, 10 mM magnesium sulfate, 0.5% sodium chloride, 0.6% agarose), and superposedly inoculated on NZYM agar medium (1% NZ amine, 0.5% yeast extract, 10 mM magnesium sulfate, 0.5% sodium chloride, 1.5% agar powder). After culturing at 37°C until the diameter of plaques reached to about 0.5 mm, the plates were left stood at 4°C for over one hour. The plaques on the plates were adsorbed on Hybond-N^+ nylon membrane (Amersham), treated with denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 7 minutes and with neutralization solution [1.5 M sodium chloride, 0.5 M Tris-HCl (pH 7.2), 1 mM EDTA] for 3minutes, and washed with 2 x SSPE. Subsequently, the DNAs were immobilized on the membranes using 0.4 M sodium hydroxide, and washed with 5 x SSPE (twice). Clones of interest were selected from 6.0 x 10^5 clones in total.

After the primary screening, PVS1, PVS3 and PVS4 were selected the bands of which were of the sizes as predicted by PCR using primers specific for each of the PVS1 to 4 members, and used in secondary and tertiary screening.

Production of probes, hybridization, washes, and autoradiography were conducted similarly as described in the section 18. "Southern Hybridization."

17. Isolation and Purification of Phage DNA

Isolation and purification of phage DNA were performed according to the method of liquid culture (See Reference 23) and polyethylene glycol (PEG) precipitation (See Reference 37) as follows. Plaques of interest were harvested from agar, transferred to 1.5 ml tubes containing 100 μl of SM solution [50 mM Tris-HCl (pH7.5), 0.1 M sodium chloride, 7 mM magnesium sulfate, 0.01% gelatin] and 1μl of chloroform, left stood at 4°C overnight, and thoroughly suspended. Using 200 ml flask, host E. coli [XLl-Blue MRA (P2) strain] was shake-cultured in 80 ml NZYM (1% NZ amine, 0.5% yeast extract, 10 mM magnesium sulfate, 0.5% sodium chloride)

at 30°C overnight. Host E. coli that had precipitated upon centrifugation (8,000 rpm, 3 minutes, 4°C)was collected and suspended in 10 mM magnesium sulfate to A_{600}=2. 500 μl of host E. coli suspension thus prepared and 50 μl of phage suspension were mixed, left stood at 37°C for 20 minutes, and shake-cultured using 50 ml NZYM at 37°C to ensure bacteriolysis. 2.9 g sodium chloride and 0.4 ml chloroform were added, and the mixture shaked for further 10 minutes. Supernatant produced by centrifugation (10,000 rpm, 10 minutes, 4°C) was collected, one fifth as much of which supernatant of 50% PEG 6000 was mixed, and the mixture left stood in ice for one hour. Precipitate produced by its centrifugation (12,000 rpm, 20 minutes, 4°C) was collected and suspended in 400μl Tris-Mg-NaCl [10 mM Tris-HCl (pH7.5), 49.6 mM sodium chloride, 4.9 mM magnesium chloride]. To the solution, 4 μl of 10 mg/ml RNase A (Sigma) and 4 μl of 10 mg/ml DNase I (Sigma) were added, the mixture treated at 37°C for one hour, and chloroform extraction performed three times. 2 x STE [80 mM Tris-HCl (pH7.5) , 2% SDS, 0.5 M EDTA] in an amount equivalent to the upper layer collected, and one fifth as much of 10 mg/ml proteinase K were added, the mixture treated at 65°C for 10 minutes and extracted in a time course by the same volume of Tris saturated phenol, phenol:chloroform:isoamylalcohol (25:24:1, v/v/v), chloroform:isoamylalcohol (24:1, v/v). To collected upper layer, twice as much of cold ethanol was added and the mixture left stood at -20°C for 30 minutes, and centrifuged (12,000 rpm, 10 minutes, 4°C) to collect precipitates. The precipitates was washed with 70% ethanol, vacuum dried and dissolved in 100 μl of H_2O.

18. Southern Analysis

Total DNAs of clones of interest were digested with selected restriction enzymes (Takara Shuzo), and fractionated by 0.8% agarose gel electrophoresis (See Reference 37). Fractionated DNA fragments were transferred onto Hybond-N^+nylon membranes (Amersham) by alkaline blotting (See Reference 36).

^{32}P labelled DNA probes were prerared by random priming (Feinberg and Vogelstein, 1983) using [a-^{32}P] dCTP (ICN Biochemicals) and Megaprime DNA Labelling systems (Amersham).

The nylon membranes with DNA adsorbed thereon were left stood in prehybridization solution [5 x Denharzt Solution (See Reference 37), 5 x SSPE (See

Reference 37), 0.5% SDS, 100 μg/ml heat-denatured salmon sperm DNA (Pharmacia)] at 42°C for over one hour, added ^{32}P laballed DNA probes, and hybridized at 42°C for over 16 hours. The membranes were then sequentially washed in 0.1% SDS-containing 2 x SSPE for 10 minutes (twice), and in 0.1% SDS-containing 1 x SSPE for 10 minutes (once). All of these washes were done at room temperature. Autoradiography was performed using X-rays film OMAT-AR (Kodak) and intensifying screen Lighting Plus (Dupont) at -80°C.

19. Preparation of Plasmid

Subcloning of the subject DNA fragments for sequencing was performed using pBluescript KS+ (Stratagene).

Total DNAs of the subject clones were digested with selected restriction enzymes (Takara Shuzo) and fractionated by 0.8% agarose gel electrophoresis (See Reference 37). The DNA fragments of interest were purified using QIAquick Gel Extraction Kit (Qiagen). Vectors that have been digested with restriction enzymes were dephosphorylated by treatment with Alkaline phosphatase E.coli C75 (Takara Shuzo) (37°C, one hour)and extracted in a time course with phenol:chloroform:isoamylalcohol (25:24:1, v/v/v), chloroform:isoamylalcohol (24:1, v/v). To collected upper layer, twice as much of cold ethanol and one twentieth as much of 3 M NaCl were added, and the mixture left stood at -20°C for 30 minutes and centrifuged (12,000 rpm, 10 minutes, 4°C) to collect precipitates. The precipitates were washed with 70% ethanol, vacuum dried, and dissolved in 20 μl TE[10 mM Tris-HCl (pH 8.0), 1 mM EDTA]. The vector and inserts prepared in the above manner were adjusted to become in molar ratio 1:1 of vector: insert, and ligated with DNA Ligation Kit ver. 2 (Takara Shuzo). E. coli JM109 Compatible Cell (Takara Shuzo) was transformed with plasmid DNA that had been ligated, inoculated on LB/Amp/X-gal/IPTG agar medium (1% Bacto Tripton, 0.5% yeast extract, 1% sodium chloride, 0.1 mg/ml ampicillin solution, 0.004% X-gal solution, 0.5 mM IPTG solution, 1.5% agar powder), and left stood at 37°C overnight. Single colonies that were obtained bay blue-white selection were cultured in LB/Amp liquid medium (2 ml LB, 0.1 mg/ml ampicillin) overnight, and their plasmid DNA isolated. Extraction and purification of plasmid DNA were performed using FlexiPrep Kit (Amersham Pharmacia Biotech).

20. DNA Sequencing and Database Analysis

Sequencing of nucleotides was performed using PRISM Dye Deoxy Termination Cycle Sequencing Ready Reaction Kit (Applied Biosystems) based on deoxy-termination method (See Reference 38). Determination of mobility of the reactant in modified polyacrylamide gel and sequencing were performed using ABI 373S DNA sequencer · DNA sequence automatic analyzer (Applied Biosystems). Binding between nucleotides, amino acid sequence in reading frame and any homology to known genes were analyzed using BLAST program (See Reference 1) on the super computers of National Institute of Genetics, DNA Data Bank of Japan (DDBJ). Alignments of amino acid sequences were performed using CLUSTAL w program (See Reference 43).

21. Transient Assay Using Potato Tuber Protoplasts

A transient assay using potato tuber protoplasts were performed as follows according to the method described in Hashimoto et al. (1992) (See Reference 18). 25 μg of transgenes were added to 800 μl of solution (0.5 M mannitol, 0.1 mM $MgSO_4$, pH 7.0) containing 1 x 10^6 protoplasts derived from potato cultured cells, gently mixed by pipetting, and left stood on ice for 10 minutes. The solution was tranferred into cuvettes that had been cooled, and electroporated with gene transfer apparatus CUY21 (Tokiwa Science) at constant current (60v, 50 pon, 75 poff, 4 times). The solution was transferred into centrifuge tubes, left stood on ice for 10 minutes, had its supernatant removed, added 900 μl of culture medium, transferred to 12-well culture plates, and left stood at 20°C in darkness for one hour. Those protoplasts into which vectors containing a deduced promoter region for PVS3 had been electroporated were added 100 μl of sterilized water or 100 μl of 1 mg/ml HWC, and left stood for 12 hours. Those protoplasts as positive control into which vectors containing a promoter region for CaMV 35S had been electroporated were left stood for 12 hours. After removing supernatant, the protoplasts were washed with 1 x PBS, and luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega).

22. Production of Transgenic Plant and Its GUS Staining

Production of transgenic plant and its GUS staining were performed according to the method of Jefferson (1987) (See Reference 19). For production of transgenic plant, pedicles of aseptically cultured MayQueen were used. CaMV35S promoter of transforming vector pBI121 (Clonetech) was deleted, deduced PVS3

promoter region 2,648 bp upstream of its translation start codon was linked upstream of GUS gene via BamHI such that GUS will be translated in-frame (Fig. 11). The vector was introduced into Agrobacterium tumefaciens LBA4404 (Clonetech) by electroporation. Ablated pedicles were immersed in culture solution of A. tumefaciens for 2 minutes for infection, and left stood on 3C5Zr medium [Sucrose 30 g, GellanGum 2 g, MS mineral (10x) 100 ml, Fe-EDTA 5 ml, Myo-inositol 100 ml, 3C5ZR vitamin (Thiamin HCl 1 mg/ml 1 ml, Nicotinic acid 1 mg/ml 0.5 ml, Pyridoxine HCl 1 mg/ml 0.5 ml, Asparatic acid 1 mg/ml 0.4 ml) 2.4 ml, IAA (0.1 mg/ml) 5.3 ml, Zeatin riboside (0.1 mg/ml) 17.5 ml, pH 5.9, per 1,000 ml] in dishes at 23°C for three days. The pedicles were then transferred to 3C5Zr medium containing kanamycin (100 μg/ml) and Cefotaxime (300 μg/ml). This step was repeated every week, until shoots bud out at which time point the pedicles were transferred into S1 regeneration medium (Sucrose 15 g, GellanGum 3 g,, S1 mineral (10x) 100 ml, Fe-EDTA 5 ml, V2 vitamin 2.0 ml, pH 5.7, per 1,000 ml) to confirm regeneration of roots.

For GUS staining, plant tissues were vacuum penetrated with GUS stain solution [X-Gluc (50 mg/ml in DMF) 100 μl, 500 mM Phosphate buffer (pH 7.0) 1 ml, 100% Methanol 2 ml, 0.5% Triton X-100 7.9 ml, per 10 ml] and stained at 37°C in darkness overnight. The stained tissues were then boiling-decolored in acetate:ethanol:glycerol (1:3:1) decoloring solution, and visualized. For microscopic study of the inoculated potato Phytophthora, the tissues stained were boiled in lactophenol solution (lactic acid 10 ml, phenol 10 g, glycerol 10 ml water 10 ml, 40 ml ethanol), and this step was repeated. Subsequently, the tissues were placed on paper filters soaked in chloral hydrate (2.5 g/ml) for two days at 4°C in darkness to destain and visualized (See Reference 49).

23. Transient Assay via Agrobacterium tumefaciens in Potato Leaf Tissue

Transient assay via A. tumefaciens was performed according to the method of Chang et al. (2002) (See Reference 7). Rifampicin (50 μg/ml) and selected antibiotics were added to A. tumefaciens LBA4404 into which binary vectors containing Cf-9/Avr9 or StMEK^{DD} (SEQ ID NO:7)had been electroporated, and the mixture cultured. The A. tumefaciens were collected by centrifugation (3,000 rpm, 15 minutes), suspended in introduction buffer (1/10x Murashige-Skoog salts, 1/10x B5 vitamins, 2% sucrose, 1% glucose, 150 μM acetosyringone, 20 mM MES pH 5.4) , and

its concentration adjusted to OD_{600}=0.1. 1 ml sylinge was used to inject the suspension from the back of the leaf, and GUS staining was performed two days later.

<Example 1> Expression Profile of PVS Gene in Potato Tuber Tissue Inoculated with P. infestans.

Potato tuber tissues were inoculated with compatible and incompatible races, water-treated, and had their total RNA extracted from three tuber discs to perform Northern analysis using PVS1 cDNA. The results of the analysis are shown in Fig. 2. Accumulation of PVS mRNA was found in both compatible and incompatible interactions.

<Example 2> Each PVS1 to 4 Member Specific RT-PCR in Potato Tuber Tissue Inoculated with P. infestans.

In order to determine which of the PVS1 to 4 members were expressed in potato tuber tissues, total RNA was extracted from three discs of potato tuber that had been inoculated with compatible and incompatible races, water-treated and frozen three or 6 hours later to perform RT-PCR using primers (SEQ ID NO: 9, 10, 13, 14, 15, 16, 17, and 18) specific for each of the PVS1 to 4 members. Bands having predicted sizes of 469 bp, 132 bp, 326 bp and 469 bp were detected for PVS1 to 4, respectively in both compatible and incompatible interactions. (Fig. 3).

<Example 3> Western Analysis of PVS Proteins in Potato Tuber Tissue Inoculated with Incompatible Race and Compatible Race

In order to determine if the accumulation pattern of PVS mRNA was reflected upon actual protein synthesis, anti-potato PVS antibody was produced to perform Western analysis. In order to obtain antigen for use in antibody production, expression in E. coli was effected based on deduced amino acid sequence. PVS1 cDNA translated region prepared by PCR was inserted into an expression vector, and expressed in E. coli as fusion protein with thioredoxin. SDS-PAGE was performed on total E. coli proteins before and after induction of expression, and the gels stained with CBB solution. As a band of approximately 83 kD in size was detected in the urea fraction, the fraction was used as antigen for antibody production after removal of urea by dialysis.

The titer of resulted antibody was determined to detect 10 ng of antigen when

used in 1,000-fold dilution. Since the antibody was thus determined to have enough titer for further use in Western analysis, soluble fractions were prepared from potato tuber discs that had been aged for 24 hours, at different time points up to 24 hours post water-treatment or inoculation with incompatible race or with compatible race, to perform Western analysis using anti-potato PVS antibody (Fig. 4). In 6 hours, accumulation of PVS proteins was found in both compatible and incompatible interactions. On the other hand, no accumulation of PVS proteins was found in water treatment area. These results may support the results of Fig. 2 that accumulation of PVS mRNA peaks 6- 9 hours after pathogen inoculation.

<Example 4> RT-PCR Specific to Each PVS1 to 4 Member in Potato Leaf Inoculated with P. infestans

In order to determine which of the PVS1 to 4 members were expressed in potato leaf tissues, total RNA was extracted from three potato leaves at different sequential time points up to 12 hours post inoculation with compatible and incompatible races, water treatment and wound treatment, to perform RT-PCR using primers (SEQ ID NO: 11, 12, 13, 14, 15, 16, 19, and 20) specific to each of the PVS1 to 4 members. Subsequently, Southern analysis was performed using cDNA probes specific to each of the PVS1 to 4 members. Only one band indicative of significant accumulation of mRNA corresponding to a predicted size of 326 bp representing PVS3 was detected in both compatible and incompatible interactions (Fig. 5). In addition, when RNA derived from tuber tissue used as positive control was used, bands of predicted sizes 176 bp, 132 bp, 326 bp and 131 bp representing PVS1-4, respectively, were detected (Fig. 5).

<Example 5> Screening of Potato Genomic Library

In view of the fact that genomic size of potato is 1.6 to 1.8 x 10^9 bp per haploid (See Arumuganathan and Earle, 1991), that average size of potato genomic library is 1.5 x 10^4 bp per plaque, and that potato is quadloid, in order to screen all of the potato chromosomes, at least 5.2 x 10^5 plaques must be screened. Thus, using full length PVS1 cDNA was used as probe to screen 6.0 x 10^5 plaques. As a result of primary screening, 87 clones were found. In order to distinguish PVS1 to 4 among the clones and obtain their deduced promoter regions, primers specific for each member of PVS1 to 4 were constructed to obtain distinct PCR products for electrophoresis. 3 clones were selected for each of PVS1, PVS3 and PVS4 and used

in further secondary and tertiary screening.

Clones obtained by the screening were separately digested with EcoRI, HindIII or XhoI, and subjected to Southern hybridization using as probes the PCR products that had been obtained with the primers used in the screening. As a result, bands that hybridized with these probes were detected. Since it was found that clones of interest were obtained, the hybridized DNA fragments obtained by EcoRI and HindIII digestion were subcloned into pBluescript KS+ vector and sequenced.

<Example 6> Determination of DNA Sequence and Database Analysis

The DNA fragments of PVS1, PVS3 and PVS4 subcloned were sequenced entirely (Figs. 6, 7, 8 and 9). In Figs. 6 and 7, promoter region (SEQ ID NO:1) and coding region (SEQ ID NO:21) were shown . In order to examine PVS3 cDNA and genomic structure of PVS3, PVS3 cDNA that had been already isolated (See Reference 53) was compared with PVS3 genomic DNA sequence obtained in the present Example. In PVS3, it was found that all of the nucleotide sequences and deduced amino acid sequences, except for 3'-untranslated region, coincide with each other, with 6 introns intervening (Fig. 8). On the other hand, both of the PVS1 and PVS4 are intervened by 5 introns, unlike PVS3 (Fig. 9). It is known that cultivars of potato plants are quadloid, with their genome having a plurality of isogenes (Reference 56). Since the PVS3 genomic clone obtained in the present Example differs from PVS3 cDNA only in 3'-untranslation region, it may encode one of PVS3 subfamilies.

Back and Chappel (1996) reported functional differentiation of sesquiterpene cyclase (See Reference 4). Deduced amino acid sequences of 5-epi-aristolochin synthase which is sesquiterpene cyclase for tobacco (TEAS), and capsicum (PEAS), and vetispiradiene synthase for henbane (HVS) and potato (PVS) were compared. Between HVS and PVS3 or PVS4, among the same VSs, identity over 90% was found in the vetipiradine specific domain (Fig. 9). On the other hand, the identity between PVS3 and PEAS was below 80%. Further, in aristolochene specific domain where substrate binding site lies, the identity between PVS and TEAS, or between PVS and PEAS were 78% to 89%, whereas the identity between PVS and HVS was found to be as high as over 98%. In addition, it was found that those sesquiterpene cyclase which are expressed in leaf tissue consist of 7 exons intervened by 6 introns, whereas those PVS1 and PVS4 which are expressed in potato tuber tissue consist of 6 exons with 5

introns intervening (Fig. 9).

<Example 7> HWC Responsiveness of PVS3 Promoters by Transient Assay Using Protoplast

pGL3 vector constructed with deduced PVS3 promoter region linked upstream luciferase was electroporated into protoplasts to examine HWC responsiveness (Fig. 10). Compared to water-treatment area, HWC treatment area had a significantly high luciferase activity, thus demonstrating that the 2,648 bp region upstream the translation start codon used in the present experiment is responsive to elicitors.

<Example 8> Expression Behavior of PVS3 Gene in Transgenic Plant

In order to closely examine any expression behavior of PVS3, binary vectors (Fig. 11) constructed with deduced PVS3 promoter region linked upstream GUS gene were transduced into MayQueen having no true resistance gene against potato Phytophthora to produce transformants. In order to examine the responsive ness of PVS3 gene to wound, a portion of the transgenic potato leaf tissue was resected and stained with GUS sequentially in a time course (Fig. 12). As a result, no GUS staining was detected at resected site even after 48 hours, thus demonstrating that the promoter of interest lacks responsiveness to wound.

In order to examine any response to P. infestans, compatible race was inoculated and microscopic observation was performed. GUS staining was observed in invaded cells within 6 hours (Fig. 13). Further, 48 hours after inoculation, an intense expression was observed on the entire leaves inoculated. These indicate that the promoter of interest is responsive to infection with compatible race of P. infestans.

In order to examine the existence of any organ constantly expressing the present promoter, an entire transgenic potato plant was GUS stained (Fig. 14). Except for Phytophthora inoculated leaf tissue used as positive control of GUS staining (Fig. 14), no staining was observed at any location such as growing points and roots. This result indicates that the present promoter is pathogen-responsive specifically.

In order to examine to which of disease signals the present promoter is

responsive, leaf tissue was treated with a variety of elicitors and stained with GUS (Figs. 15, 16, 17 and 18). It was found that when treated with arachidonic acid which is a constituent fatty acid of P. infestans, the tissue was GUS-stained in 24 hours (Fig. 15). On the other hand, when treated with hydrogen oxide which is one of reactive oxygen species, or with glucose/glucose oxidase which produces hydrogen peroxide, or with salicylic acid which is involved in systemic acquired resistance, the tissue was not GUS-stained (Figs. 16, 17 and 18).

It is well known that when resistance gene product Cf-9 of a tomato form responds to corresponding Avr9 which is a specific elicitor of tomato leaf fungus Cladosporium fulvum, communication mechanism is triggered, thereby inducing hypersensitive reaction (See Reference 41). When Cf-9/Avr9 was transiently expressed via Agrobacteria in leaf tissue, GUS staining resulted (Fig. 19). Further, when constitutive active mutant enzyme $StMEK^{DD}$ (SEQ ID NO:7, 8) which phosphorylates and activates salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK) known to be downstream of these resistance genes and control a variety of protective responses was expressed, GUS staining similarly resulted (Fig. 19). On the other hand, in leaf tissue inoculated with Agrobacteria containing the binary vector as control without any insert, no GUS staining was found.

HMG-CoA reductase (HMGR) genes that play an important role in rishitin synthesis constitute a multigene family (Fig. 1). HMG1 contributes to steroid glycoalkaloid synthesis in response to wound, whereas HMG2 and HMG3 are known to be induced by pathogen signal and contribute to rishitin synthesis (See Reference 9). It is reported that PVS genes in potato plants also constitute a multigene family, and that there are PVS1 to 4 members (See Reference 53). In the present study, in order to examine expression pattern of PVS 1 to 4 members in potato tuber tissue and leaf tissue, RT-PCR was performed using primers specific for each of these members. Total RNA extracted from potato tuber discs that had been inoculated with compatible and incompatible races of P. infestans was used as template in performing the RT-PCR (Fig. 3). Regardless of which race had been inoculated, bands were detected which correspond to predicted sizes of all of PVS1 to 4 members. These results may indicate that, at least in tuber tissue, the PVS members may not have distinctive roles for metabolic fluctuation in response to stimuli.

In order to examine whether the accumulation pattern of PVS mRNA is reflected upon PVS protein synthesis in potato tuber tissue, anti-potato PVS antibody was produced to perform Western analysis (Fig. 4). Accumulation of PVS proteins was found 6 to 24 hours after inoculation with incompatible race and compatible race. It has been reported, as a result of Northern analysis using total RNA extracted from potato tuber tissue, there was a transient accumulation of PVS mRNA that peaked 6 to 9 hours after inoculation in areas where incompatible and compatible races had been inoculated (Fig. 2, Reference 53). In view of the accumulation profile of PVS mRNA, the half-life of PVS proteins is expected to be long. Further, it has been reported that PVS enzyme activity increases in soluble fraction prepared from potato tuber tissue inoculated with either of incompatible race and compatible race (See Reference 54). This report contradicts another report that accumulation of phytoalexin is induced only by inoculation with incompatible race (See Reference 40). For phytoalexin biosynthesis in potato, HMGR enzyme, which biosynthesizes mevalonic acid from 3-Hydroxy-3-methylglutaryl CoA (HMG-CoA), and PVS enzyme, which biosynthesizes vetispiradiene from farnesyl diphosphate, are thought to be two major factors (See References 29, 54, and 9) (Fig. 1). It is reported that HMGR activity is significantly increased only in incompatible interaction, whereas it is decreased with time in compatible interaction (See Reference 52). In view of the activity profile of HMGR, the specific phytoalexin synthesis control between potato cultivars and physiological races of P. infestans is determined by supply of mevalonic acid. In order to confirm this scenario, it may be necessary to examine whether or not those phytoalexins in the forms of lubimin and rishitin are accumulated by externally supplying tuber tissues that had been inoculated with compatible race with farnesyl diphosphate, which is substrate of PVS.

The first actual tissue infected with P. infestans is leaf tissue. In the present study, in order to examine which of PVS1 to 4 members are expressed by attack of P. infestans, total RNA extracted from leaf tissue in a time course after inoculation with compatible and incompatible races and treatment with water was used as template to perform RT-PCR (Fig. 5). In both compatible race and incompatible race interactions, it was found that only PVS3 was significantly induced. It is known in general that rishitin does not accumulate in potato leaf tissues (See Reference 34). However, an observation has been reported that rishitin is synthesized transiently also in leaf tissues

(See Reference 26). Although the role of rishitin in leaf tissues in protective response is not yet known, the fact that PVS3 is induced by inoculation of compatible race allows construction of disease by resistance plant utilizing the present promoter. Thus, in order to obtain PVS3 promoter, genomic clone was isolated. As a result of screening, PVS1, PVS3 and PVS4 were obtained (Figs. 6, 7, 8, 9). Comparison between the PVS3 genomic clone isolated this time and PVS3 cDNA that has been already isolated revealed a coincidence across all of the deduced amino acid sequences. Restriction enzyme sites in exon regions agreed with each other in a corresponding manner (Fig. 8).

Capsidiol, which is a phytoalexin for tobacco and capsicum, and rishitin produced by henbane and potato are synthesized by similar biosynthesis pathways (See Reference 4 and 29) (Fig. 1). Sesquiterpene cyclase involved in the former is 5-epi-aristolochin synthase (EAS), and EAS for tobacco (TEAS) and capsicum (PEAS) are extremely similar in amino acid level (See Reference 53). Back and Chappell constructed various chimeric genes using TEAS and HVS cDNA for henbane, and synthesized proteins from chimeric genes in E. coli (See Reference 4). By adding farnesyl diphosphate, which is a substrate for EAS and VS, to soluble fraction of the recombinant proteins, and by quantifying production ratio of 5-epi-aristolochin or vetispiradiene, any region controlling the activity of both enzymes was predicted. Comparison of deduced amino acid sequences defined by each exon according to their report revealed, in vetipiradiene specific domain, over 90% identity between HVS of henbane and PVS4 or PVS3. In aristolochene specific domain where substrate binging site lies, identity of 78% to 89% was found between PVS and TEAS or PEAS, whereas identity of over 98% was found between PVS and HVS (Fig. 9). This result supports the theory asserted by Back and Chappell (See Reference 4). It was further found that PVS1 and PVS4 consist of 6 exons intervened by 5 introns, whereas PVS3 and other sesquiterpene cyclase that are expressed in leaf tissue consist of 7 exons intervened by 6 introns (Fig. 9). Miyata (1984) assumed that those introns in mitochondria genome that lacked any function to facilitate an efficient replication are deleted, thereby shortening DNA in the process of evolution. Based on his theory, it may be envisioned that the fifth intron of PVS1 and PVS4 expressed in tuber is a product of deleted intron and shortening in the process of evolution.

For creation of disease resistant plant utilizing a promoter, it may be

necessary to analyze and identify PVS3 promoter region. In the present Example, GUS gene was linked downstream of deduced promoter of PVS3 to produce transgenic potato plant and closely examine the responsiveness of the present promoter. Interestingly, the present promoter was not only unresponsive to wound (Fig. 12), but it also caused no staining either at any growth point or root (Fig. 14). There is a report of construction of transgenic tobacco plant carrying GUS gene similarly linked downstream of TEAS, sesquiterpene cyclase of tobacco plant, and of its expression pattern (See Reference 51). The researchers reported, even at a low level, responsiveness to wound, and GUS activity in roots and pedicles. According to this report, PVS3 promoter in potato leaf tissue and TEAS promoter in tobacco leaf tissue may have distinct manner of response. In tobacco leaf, capsidiol, a type of phytoalexin, is accumulated to a high concentration in response to pathogen attack and elicitor treatment. In contrast, there is no rishitin accumulation found in potato leaf tissue, and PVS3 mRNA accumulation is at so low a level as to be detected by RT-PCR (Fig. 5). Based on these facts, the specific responsiveness of PVS3 promoter may attribute to the low level of expression.

Recently, it has been reported that HMGR gene expression is controlled by SIPK, a type of mitogen-activated protein (MAP) kinase (See Reference 33). Also in the present study, when constitutive active mutant enzyme $StMEK1^{DD}$ (See Reference 50) was expressed which phosphorylates and activates SIPK and WIPK, GUS activity was found (Fig. 19). Further, when Avr9, which is a specific elicitor by tomato leaf fungal disease fungus Cladosporium fulvum, and Cf-9, which is a resistance gene product of tomato cultivar, were transiently expressed in leaf tissue via Agrobacteria, GUS staining resulted (Fig. 19). Romeis et al. reported that when those tobacco plant and cultured cell which have transformed Cf-9 were treated with Avr9, SIPK and WIPK were activated (See Reference 35). Based on these findings, PVS3 promoter may be controlled by SIPK, similarly to HMGR gene. This inference may be supported by the fact that treating potato tuber tissue with HWC or arachidonic acid activates MAP kinase corresponding to SIPK (See Reference 20). A predicted communication pathway behind these events is shown in Fig. 20.

MAPK cascade is one of the most important factors of signal transduction pathway in plants and has drawn an intense focus in recent years (Reference 65). SIPK and WIPK, among others, have been implicated in expression of disease

resistance in plants (Reference 57). MAPKKK located upstream of MAPK cascade is known to phosphorylate and activate MAPKK, which in turn phosphorylates MAPK, thereby triggering a variety of protective responses. It has been recently shown that when Benthamiana (Nicotiana benthamiana), a type of tobacco, was caused to overexpress a constitutive active mutant enzyme $StMEK1^{DD}$ for StMEK1, a MAPKK, SIPK and WIPK were activated to induce 5-epi-aristolochin synthase (TEAS), a sesquiterpene cyclase of tobacco plant (Reference 64). It is readily expected that MAPK cascade is involved also in potato PVS gene regulation, similarly to tobacco plant. In the following Example, virus-induced gene silencing (VIGS) was used to explore this possibility. VIGS has come into focus in recent years as effective gene knock down method useful in analyzing function of plant genes (Reference 59). VIGS is one of biophylaxis against viruses wherein transcription product of any host gene that has a high homology with viral gene is specifically degraded. Since those mRNA which are 80% or more homologous with any plant gene fragment introduced into virus is degraded, in general, this method is useful for knocking down not only one sole gene but also its multi-gene family. This method can also be readily and quickly performed, compared to production of transformant or mutant. Above all, numerous studies have been reported concerning potato X virus (PVX) and Benthamiana system as method for retrospective genetic functional analysis (References 67 and 70). Hence, SIPK and WIPK were knocked down by VIGS, and the above described PVS3:GUS transiently introduced into leaf tissue via Agrobacteria to examine PVS3 promoter activity.

In the following Examples 9 through 12, biological materials, reagents, experimental procedures and other details are as follows. Unless otherwise specified, materials and other items are similar to those used in the above Examples.

1. Tested Plant

As tested plant, Benthamiana (Nicotiana benthamiana) provided by Leaf Tobacco Research Center, Japan Tobacco, Inc., was used. Benthamiana was seeded on Kureha soil (Kureha Chemicals) contained in polyethylene pots, which were then placed in an incubato at 25°C to develop the plant under light for 24 hours. For transient assay using Agrobacterium (Agrobacterim tumefaciens), 6 through 8 leaves from plants 30 through 35 days post seeding were used.

2. Preparation of INF1

As Infestin with which to treat Benthamiana leaf, a fusion protein expressed in E. coli (Escheruchia coli strain pBF53) having FLAG-ATS vector comprising inf1 gene introduced was used (Reference 62). The fusion protein was prepared as follows.

Each E. coli was shake-cultured (140 rpm) overnight in LB liquid medium containing 50 μg/ml ampicillin at 37 °C. The E. coli culture medium was added to LB liquid medium containing 100-fold amount, i.e. 50 ppm, of ampicillin, and shake-cultured (140 rpm) at 37 °C further to the optical density of $OD_{600} = 0.6$. IPTG was added to the final concentration of 1 mM to induce expression of protein, shake-cultured (140 rpm) for three hours at 37 °C, and then centrifuge the culture medium (5,000 x *g*, 10 minutes). Supernatant was filtrated, transferred to dialysis tubes (exclusion limit molecular weight 3,500), and dialyzed against sterile distilled water at 4 °C for 24 hours. Fraction thus obtained was adjusted to the protein concentration of 10 mg/ml, to provide FLAG-INF1 solution. For use in treating plant, the INF1 solution was diluted 3-fold with distilled water.

3. Construction of Binary Vector harbouring Inserted PVS3 Promoter with GUS Gene Linked.

Expression vector for use in MUG assay was constructed as follows. Using PVS3 genomic clone as template, primers with restriction enzyme sites (*Eco*RI or *Cla*I) added were used (Fig. 24) to amplify nucleotide sequence comprising deduced promoter region and PVS3 gene coding region start site by PCR. The PCR reaction was performed using KOD -Plus- DNA Polymerase (Toyobo), with annealing temperature of 55°C, according to an attached protocol. The PCR product was digested with *Eco*RI and *Cla*I, fractionated by electrophoresis on 1% agarose gel, and DNA fragments of interest purified with QIAquick Gel Extraction Kit (Qiagen) and used as insert. As vector, pGreen 0229 (See Hellens et al. 2000) including GUS gene comprising introns was used, digested with *Eco*RI and *Cla*I, and fractionated by eletrophoresis on 1% agarose gel, similarly to the insert. DNA fragments of interest were purified using QIAquick Gel Extraction Kit and used as vector. The vector and insert thus prepared were adjusted to 1:3 in vector:insert in molar ratio, and ligated with DNA Ligation Kit ver. 2 (Takara). E.coli JM109 Compatible Cell (Takara) was transformed with ligated plasmid DNA, inoculated on LB agar medium [1% tryptone

peptone, 0.5% yeast extract powder, 1% NaCl, 50 μg/ml kanamycin, 1% agarose] , and cultured at 37 °C overnight. Single colonies were cultured on 2 ml LB liquid medium [1% tryptone peptone, 0.5% yeast extract powder, 1% NaCl, 1% agarose] containing kanamycin solution (50 μg/ml) at 37 °C overnight, and plasmid DNA recovered. Map of binary vector with PVS3 promoter inserted is shown in Fig. 25.

4. Construction of Deletion Clone for Use in Transient Assay in Benthamiana Leaf

Using as template the GUS expression vector constructed in the section 3 above, primers with added restriction enzyme sites (*Eco*RI or *Cla*I) starting with deletion points of interest (Fig. 24) were used to amplify nucleotide sequence comprising deduced promoter region and PVS3 gene encoding region starting site by PCR. This PCR was performed using KOD -Plus- DNA Polymerase (Toyobo), with annealing temperature of 55°C , according to the attached protocol. Binary vector was prepared according to the procedure described above in the section 3, and introduced into Agrobacteria according to the method described in the section 6. Deletion clones obtained were confirmed to be free of error in nucleotide sequence.

5. Construction of StMEK1^{DD} Expression Vector

Expression vector for use in MUG assay in Benthamiana was constructed as follows. Nucleotide sequence of StMEK1^{DD} (See Reference 64) comprising 5' untranslated region was amplified by PCR using primers (5'-TTGGGCCCATGCGACCTCTTCAACCACC-3' : SEQ ID NO: 36, 5'-GACTAGTACAAAAGAGTGTGGAATTAC-3' : SEQ ID NO:37) with restriction enzyme site (*Apa*I or *Spe*I) added. This PCR was performed using KOD -Plus- DNA Polymerase (Toyobo), with annealing temperature of 55 °C, according to the attached protocol. The PCR products were digested with *ApaI* and *SpeI* and fractionated by electrophoresis on 1% agarose gel, DNA fragments of interest purified using QIAquick Gel Extraction Kit (Qiagen), and used as insert. As vector, pER8 (Reference 74) wherein transgene is induced by ß-estradiol to express was used, digested with *Apa*I and *Spe*, and fractionated by electrophoresis on 1% agarose gel, similarly to the insert. DNA fragments of interest were purified using QIAquick Gel Extraction Kit and used as vector. Following procedures were performed according to the ligation step in the section 3 above.

6. Transformation of Agrobacterium

Vector for introduction was adjusted with TE to 10 ng/μl and used for transformation of Agrobacterium. Binary vector was constructed as follows. Compatible cells of 80 μl Agrobacterium GV3101 strain were fused on ice, to which 2 μl vector solution was added, mixed by pipetting, and left stood on ice for 30 minutes. This solution was transferred to cuvette, and electroporated (V = 1.44 kV, T = 2.5 kV/resistance, C = all out, R = R5 129) with Micro Pulser™ (Bio-Rad) to transform. The solution was then transferred into 1.5 ml Eppendorf tube, added to 1 ml of SOC medium [2% tryptone peptone, 0.5% yeast extract powder, 0.05% NaCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$] and left stood at room temperature for one hour. The resultant solution was inoculated on LB agar medium [1% tryptone peptone, 0.5% yeast extract powder, 1% NaCl, 50 μg/ml kanamycin, 50 μg/ml rifampicin, 1% agarose] , and cultured at 28 °C for two days. Single colonies were recovered and used for Agroinfiltration Experiment in the section 7 below.

7. Gene Transfer by Agroinfiltration Using Benthamiana Leaf

According to the method described by Thomas et al. (2000) (See Reference 41), Agroinfiltration was performed as follows. Agrobacteria harbouring binary vectors introduced were shake-cultured in LB liquid medium containing a selected antibiotic at 28 °C for two days. 2 ml of the culture solution was suspended in 8 ml LB liquid medium containing an antibiotic, and shake-cultured at 28 °C for further 3 hours. Density of Agrobacteria in the suspension was measured using a ultraviolet visible spectroscopic analysis system (DU series 600, Beckman) and calibrated by absorbance at wavelength 600 nm. The suspension was centrifuged (3,000 x *g*, 15 minutes) at room temperature, and precipitates resuspended in 10 mM MES (pH 5.6) containing 150 μM acetonitocilingon, 10 mM $MgCl_2$ to OD_{600} = 0.5.

For Agroinfiltration of Agrobacteria retaining PVS:GUSint only, suspension of OD_{600} = 0.5 was injected into leaves, and 10 μg/ml INF1 solution was injected one day later to induce PVS3 promoter (Fig. 26). On the other hand, for Agroinfiltration of Agrobacterium retaining XVE:StMEK1^{DD} or PVS:GUSint using pER8 vector, each of their solution were diluted to OD_{600} = 0.005 and OD_{600} = 0.25, respectively, XVE (LexA, VP16, estrogen receptor) system having 20 μM ß-estradiol linked downstream of G10-90 promoter was induced to express StMEK1^{DD} (Reference 74). As control area, pER8 vector, intead of XVE:StMEK1^{DD}, was used to inject 20 μM ß-estradiol (Fig. 26).

The suspension was left stood at room temperature for one hour, and then injected into intercellular spaces of Benthamiana leaves using needless syringe. The plant after injection was left stood at 25°C for 24 hours, and used for MUG (4-methylumbelliferyl ß-D-glucuronide) assay described in the section 9 below.

8. Virus-Induced Gene Silencing in Benthamiana

It is known that when any nucleotide sequence within the virus with which plant infected contains any sequence homologous with any gene of the plant, virus-induced gene silencing results (Reference 67). In the present Example, PVX and Benthamiana systems having a stable incidence of gene silencing were used. To pGR106 which is a binary vector having PVX, any one of cDNA fragment of 230 bp from translation starting codon of SIPK, cDNA fragment of 178 bp from translation starting codon of WIPK, or cDNA coding SIPK and WIPK linked in tandem, was inserted to obtain gene silencing vector, which was then introduced into Agrobacterium. The Agrobacterium was cultured according to the method described in the section 7 above, and injected into Benthamiana leaves that have grown 3 weeks after seeding. The Benthamiana was allowed to grow one month and then had their upper leaves used for experiment (Fig. 27).

9. MUG Assay

MUG (4-methylumbelliferyl ß-D-glucuronide)assay was performed according to the method of Gallagher (1992) (See Reference 60) in order to quantify GUS expression. 1 cm^2 pieces of three Benthamiana leaves that had been injected with Agrobacterium were ground in liquid nitrogen, and 200 μl extraction buffer [50 mM $NaHPO_4$ (pH 7.0), 10 mM ß-mercaptoethanol, 10 mM EDTA, 0.1% sodium lauroyl sarcosine, 0.1% TritonX-100] added. The resultant solution was centrifuged (12,000 rpm, 4°C, 5 minutes) and supernatant recovered. Protein concentration of the recovered extraction was measured according to the protein quantification method described in the section 13 above, and 10 μl extraction was added to 90 μl fluoro metric buffer [extraction buffer, 2 mM MUG] at 37°C to produce 100 μl reaction solution, which was left stood at 37°C for one hour. The reaction solution was added to 900 μl quenching solution (0.2 M Na_2CO_3) for use in measurement. Using fluorospectrophotometer RF-5300PC (Shimadzu), emission spectrum at 455 nm was measured with excitation set at 365 nm. Calibration curve was generated using

4-MU (7-hydroxy-4-methylcoumarin), and measurement scaled at 4-MU nM/min · mg protein. In order to exclude any endogenous GUS activity from measurement, samples having its endogenous enzyme heat-denatured were prepared, and the balance between calibrated values were determined to calculate GUS activity derived from expression vector.

10. Extraction of Total RNA from Benthamiana Leaf

Total RNA was extracted from Benthamiana leaf based on SDS/phenol method according to the following procedure. 1 g of Benthamiana leaf was ground in mortal under added liquid nitrogen, added to 50 ml volume sterile centrifuge tubes containing extraction buffer (EB)[50 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, 5 mM EDTA, 5% SDS] 5 ml, PCI [phenol /chloroform/isoamylalcohol (50: 49:1, v/v/v)] 0.4 ml, 10 μl of mercaptoethanol 10μl , vigorously mixed for one minute, to which PCI 4.8 ml was added followed by gentle agitation. This solution was ground with polytron type homogenizer (HG30, Hitachi) for two minutes, and centrifuged (1,300 x *g*, 15 minutes). Aqueous phase (upper phase) was transferred into new 50 ml volume centrifuge tubes, to which PCI 6 ml added, agitated two minutes, and again centrifuged (1,300 x *g*, 15 min.) at ordinary temperature. To aqueous phase (upper phase) 1/40 amount of 4 M sodium chloride and 2-fold amount of ethanol was added and mixed, the resultant mixture left stood at -20°C for over 2 hours, and centrifuged (1,300 x *g*, 15 minutes). To precipitates obtained, 2 ml of resuspension buffer (RB)[50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.5% SDS] was added, and the mixture gently shaken for 15 minutes to achieve suspension. To this suspension, 0.2 ml 4 M sodium chloride and 4 ml ethanol was added, and the suspension left stood at -20°C for over two hours, and centrifuged (1,300 x *g*, 15 minutes). Resultant precipitates were washed with cold 1ml 70% ethanol, suspended in 1 ml TE buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA] , transferred into Eppendorf tubes, to which 250 μl of 10 M lithium chloride was added, and left stood on ice for one hour. The suspension was centrifuged (22,000 x *g*, 15 minutes) at 4°C, and RNA recovered as precipitates. . This lithium precipitation procedure was repeated twice, resultant precipitates suspended in 300 μl TE buffer, to which 100 μl chloroform/isoamylalcohol (24:1, v/v)was added, vigorously agitated , and centrifuged (22,000 x *g*, 15 minutes) at 4°C. To aqueous phase (upper phase), 1/10 amount of 3 M sodium acetate (pH 5.2) and 2-fold amount of ethanol were added, resultant mixture left stood at -20°C for over 2 hours, and centrifuged (22,000 x *g*, 5 minutes). Resultant precipitates was washed in cold 70%

ethanol, air-dried for 10 minutes, suspended in 40μl TE buffer to obtain total RNA, which was then stored at -80°C as RNA sample.

11. Northern Analysis

The total RNA was fractionated by formaldehyde agarose gel eletrophoresis (Reference 37), and transferred and to immobilized on Hybond-N^+ nylon membrane (Amersham) by alkaline blotting (Reference 68).

The nylon membrane with RNA adsorbed thereon was placed in prehybridization solution [50% formamide, 5 x Denhartz solution (Reference 37), 5 x SSPE (Reference 37), 0.5% SDS, 100 μg/ml heat-denatured salmon sperm DNA (Pharmacia)] at 42°C for over one hour, to which ^{32}P-labelled DNA probe was added, and allowed hybridization at 42°C for over 16 hours. The membrane was sequentially washed in 0.1 % SDS-containing 4 x SSPE at room temperature for 15 minutes (twice), 0.1% SDS-containing 4 x SSPE at 60°C for 15 minutes, and 0.1% SDS-containing 2 x SSPE at 60°C for 15 minutes (once). Autoradiography was performed using X-ray film OMAT-AR (Kodak) and intensifying screen Lighting Plus (Dupont) at -80°C.

12. Production of Probes

Using plasmid pTEAS (Facchini and Chappel, 1992) having tobacco TEAS cDNA incorporated as temperate, TEAS cDNA fragments were amplified by PCR using primers (5'-GTCGACGACACAGCCACGTACGAGGT-3' : SEQ ID NO: 38, 5'-ATCGATAGACTTTCTCCGGATGAGTG-3' : SEQ ID NO: 39). Reaction was performed using 2 ng plasmid having TaKaRa Taq^{TM}(Takara Shuzo) and an insert DNA incorporated, on DNA thermal cycler (PJ2000, Perkin Elmer Cetus) at 94°C for one minute (heat-denaturation), at 53°C for 45 seconds (annealing), at 72°C for 2 minutes (DNA elongation), in 25 cycles. The size of DNA fragments amplified by 0.8% agarose electrophoresis was determined. The DNA fragments were purified from the gel using QIAquick Gel Extraction Kit (Qiagen). ^{32}P -labeled DNA probes were produced using Random Priming method (Reference 17) using [a-^{32}P] dCTP (111 TBq/mmol, ICN Biochemicals) and Megaprime DNA labelling system (Amersham).

<Example 9> Response to INF1 by PVS3 Promoter Deletion Clone Introduced by Agroinfiltration

INF1, P. infestans-derived elicitor protein, is an effective elicitor for Benthamiana (Reference 63). Binary vectors containing GUS gene including introns linked in-frame downstream of PVS3 promoter were introduced by Agroinfiltration into Benthamiana leaves to examine INF1-induced GUS activity (Fig. 27). When binary vectors pPVS3-1 containing full length PVS3 promoter inserted were introduced, a significant increase of GUS activity was observed compared to water treatment (Fig. 28). In order to examine cis-sequence of PVS3 promoter responsice to INF1, deletion clones were produced to construct binary vectors and examine GUS activity. As a result, deletion up to -1,337 (pPVS3-2 : SEQ ID NO: 22) retained INF1 responsiveness, whereas deletion up to -1,287 (pPVS3-3) greatly reduced GUS activity induced by INF1. This result indicates that cis-sequence of PVS3 promoter is involved in 50 bp (SEQ ID NO: 23) between pPVS3-2 and pPVS3-3 (Fig. 29).

<Example 10> Induction of PVS3 Promoter by StMEK1^{DD}

StMEK1^{DD} is a constitutive active mutant produced by amino acid substitution of MAPKK, and found to induce SIPK and WIPK when introduced by Agroinfiltration into Benthamiana leaf (Reference 64). In order to determine whether or not the region of PVS3 promoter region that is responsive to INF1 treatment is similarly responsive to StMEK1^{DD}, binary vectors containing GUS gene including introns linked to PVS3 promoter were introduced by Agroinfiltration into Benthamiana. At the same time, the leaves were co-infected with Agrobacteria transformed with binary vectors which have StMEK1^{DD} linked downstream of XVE induced by ß-estradiol, and ß-estradiol injected. The leaves were then left stood for one day to allow expression of StMEK1^{DD} to examine GUS activity (Fig. 27). As a result, deletion up to -1,337 (pPVS3-2 : SEQ ID NO:22) produced an induction of greater GUS activity by injection of ß-estradiol compared to control area (Fig. 30). On the other hand, deletion of PVS3 promoter up to -1,287 (pPVS3-3) resulted a significant decrease of GUS activity induced by ß-estradiol treatment. This result indicates that the 50 bp (SEQ ID NO:23) between pPVS3-2 and pPVS3-3 is involved in cis-sequence responsive to StMEK1^{DD}, and that it is similar to that cis-sequence for INF1 (Fig. 29).

<Example 11> Effect of SIPK and WIPK Silencing on PVS3 Promoter Activity Induced by StMEK1^{DD}

pPVS3-1 was introduced by Agroinfiltration into Benthamiana silenced either

SIPK or WIPK alone, or both of SIPK and WIPK, to examine GUS activity. The Benthamiana was co-infected by Agroinfiltration with $StMEK1^{DD}$ that is linked downstream of XVE. One day after the infection, ß-estradiol was injected, the leaves left stood for one day to allow expression of $StMEK1^{DD}$ (Fig. 31). Compared to control plant infected with an empty PVX vector, the SIPK or WIPK silenced plant showed no significant reduction of GUS activity (Fig. 32). On the other hand, VIGS of both SIPK and WIPK showed a significant reduction of GUS activity.

<Example 12> Effect of SIPK and WIPK Silencing on TEAS Expression Induced by $StMEK1^{DD}$

In order to examine the control mechanism of TEAS which is tobacco sesquiterpene cyclase, expression vectors containing $StMEK1^{DD}$ gene linked to 35S promoter were introduced by Agroinfiltration into SIPK and/or WIPK silenced Benthamiana ~~silenced~~, and total RNA extracted in a time course. Northern analysis using TEAS cDNA as a probe revealed that VIGS of both SIPK and WIPK resulted in a significantly lesser accumulation of TEAS mRNA (Fig. 32). This result indicates that SIPK and WIPK have similar roles for PVS3 promoter activity.

As shown in the above Examples, in order to explore any promoter region that is important for PVS3 gene expression, PVS3 promoter was linked upstream GUS gene to construct a deletion clone, which was then treated with INF1 to measure GUS activity (Fig. 28). Deletion pPVS3-2 (SEQ ID NO: 22) showed a significant increase in activity provided by INF1 treatment, whereas deletion pPVS3-3 showed no increase in activity (Fig. 28). Gene transcription is known to be induced when transcription factor protein produced by stimuli is bound to cis-sequence within promoter region. Therefore, cis-sequence that is bound by transcription factor is thought to lie between deletion pPVS3-2 and pPVS3-3. Presently, any known control motif has not been found in this 50 bp region (SEQ ID NO:23) (Fig. 29).

It has been shown that SIPK, which is MAPK for tobacco, controls expression of 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) which plays an important role in sesquiterpenoid phytoalexin synthesis (Reference 73). It is known that MAPK cascade plays an important role in controlling signal transduction in plant, and activates a variety of protective responses located downstream (Reference 71). Moreover, it has been recently shown that when expression vector carrying $StMEK1^{DD}$

gene linked downstream of 35S promoter is introduced by Agroinfiltration into Benthamiana, TEAS is induced at the transcriptional level (Reference 64). In order to determine whether or not the region of PVS3 promoter that is responsive to INF1 treatment is similarly responsive to StMEK1^{DD}, deletion clone and StMEK1^{DD} were co-expressed by Agroinfiltration in Benthamiana leaf to examine GUS activity. Similarly to INF1, deletion pPVS3-2 showed a significant increase in activity provided by StMEK1^{DD}, whereas deletion pPVS3-3 showed no significant increase in activity (Fig. 30). Zhang et al. (1998) reported that treatment of cultured cells of tobacco with cryptogein elicitin produced by Phytophthora cryptogea or with parasiticein produced by *P. parasitica* results in activation of SIPK and WIPK (Reference 72). Taking the result of the present experiment into consideration, it may be demonstrated that MAPK cascade may be involved in the induction process of PVS3 via signal transduction by INF1 elicitin produced by P. infestans. In order to investigate this possibility, binary vector containing PVS3 promoter was introduced into SIPK or WIPK alone, or both SIPK and WIPK genes silenced Benthamiana leaves by Agroinfiltration, followed by INF1 elicitor treatment to examine GUS activity (Fig. 32). Compared to PVX inoculated control plant, WIPK or SIPK silenced plant showed a little reduction in GUS activity. On the other hand, both SIPK and WIPK silenced plant showed a significant reduction in GUS activity. These results suggest that TEAS, which is an endogenous sesquiterupene cyclase, is also controlled by SIPK and WIPK. In fact, when StMEK1^{DD} gene was expressed in SIPK and/or WIPK silenced Benthamiana, TEAS mRNA accumulation was significantly suppressed only in the area where both SIPK and WIPK were silenced (Fig. 32).

Samuel and Ellis (2002) reported that exposure of tobacco plant to a high concentration of ozone activates SIPK and WIPK, thereby inducing cell death accompanied by production of reactive oxygen species (Reference 69). They found that exposure of transgenic plant showing SIPK silencing by RNAi (RNA interference) to ozone causes a significant increase in WIPK activity, resulting in induction of cell death. In view of this report, SIPK and WIPK may compensate each other to control the expression of downstream PVS3 gene.

This invention is not limited in any way by the embodiments nor Examples described above, but encompasses any modification thereto a skilled person would think of without departing the scope of the present invention as claimed.

INDUSTRIAL APPLICABILITY

Pathogen-responsive promoter according to the present invention is applicable to expression of any desired gene specifically at the time of pathogen infection in plant cells. Accordingly, by employing any gene involved in protective response, for instance, it allows creation of pathogen-resistant plant which has a prompt protective response to pathogen infection.